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THE ROLE OF ANTERIOR PITUITARY HORMONES IN CONTROLLING SIZE, WORK AND STRENGTH OF THE HEART

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It is known that hypophysectomy prevents the development of cardiac hypertrophy (Hajdu & Beznák, 1945; Beznák, 1955*a*). In an attempt to decide which hormones of the pituitary are necessary for the development of cardiac hypertrophy, growth-hormone (GH) preparations were encountered that were effective, while others similarly labelled had no effect (Beznák, 1954*b*, 1956). Similar discrepancies were noted by White (1955) regarding the nephrotropic action of different growth-hormone preparations. Since the experiments could rarely be repeated owing to lack of the same preparation, and in many cases no detailed analysis of other hormone impurities was available, the cause of the divergent results could not be elucidated. The question was recently reinvestigated because an opportunity arose to obtain several well defined fractions of the same pituitary starting material.

Bigland & Jehring (1951) found that although treatment with growth hormone caused the mass of the quadriceps muscle of normal rats to increase, the tension developed by the muscle diminished. In view of the importance of these results an attempt was made to measure output, work and strength of the heart and to decide whether these parameters and cardiac size always changed together or whether they could be influenced separately by the various hormones.

In recent experiments Beznák (1959) analysed the haemodynamic changes following hypophysectomy. The hypotension was found to be due to a greatly diminished cardiac output, while peripheral resistance was rather higher than in normal rats. If the load on the heart was increased—the input load by infusing polyvinyl-pyrrolidone into the right heart and the resistance load by intravenous infusion of Pitressin (vasopressin; Parke Davis) or norepinephrine—the output and work of the heart rose greatly, both in normal as well as in hypophysectomized rats. It was concluded that the low cardiac output after hypophysectomy was not due to a weakness of the heart, but was the consequence of a diminished venous

return. This in turn was probably due to the reduced metabolism, as shown by the decreased oxygen consumption of hypophysectomized rats. Since it was shown that the rat heart is capable of undergoing quick changes in mass (Beznák & Hajdu, 1945; Hall, Hall & Ogden, 1953), Beznák (1959) considered that the atrophy of the heart occurring after hypophysectomy (McQueen-Williams & Thomson, 1940; Hajdu & Beznák 1945) was a sign of adaptation to the lower demand. If cardiac atrophy was only the consequence of a lower demand, preparations increasing the latter should prevent it. Eartly & Leblond (1954) found that thyroxine treatment of hypophysectomized rats restored to normal the oxygen consumption, heart rate and the heart-weight : body-weight ratio. That the latter is higher in thyroxine-treated hypophysectomized rats than in untreated controls had already been shown by De Granpré & Raab (1953) and by Scow (1954). The problems that the present investigation aimed to clarify were: Is it the thyroid-stimulating hormone (TSH) that prevents cardiac atrophy of hypophysectomized rats by the above mechanism? Are only preparations that cause a rise in oxygen consumption effective, or can atrophy be prevented when the oxygen consumption of the animal remains low? When the demand on the heart is increased by aortic constriction, are the same hormones that prevent cardiac atrophy needed for the production of hypertrophy?

METHODS

All experiments were performed on male rats of the Wistar strain weighing 160–220 g. Hypophysectomy was carried out, and beginning on the day of the operation the rats were treated for 15 days with the preparation in question as a daily intraperitoneal injection in 0·5 ml. saline. After 15 days the descending aorta was constricted in some of the rats just below the diaphragm with a silver ring of 0·8 mm diameter. After cardiac hypertrophy had developed, different determinations were carried out. Hypophysectomy and aortic constriction were performed under ether anaesthesia, the final determinations under pentobarbitone (Nembutal; Abbott Laboratories, Ltd.).

Series I was a pilot experiment. The preparation used was a lyophilized extract of whole sheep pituitaries, pH 5·5, called subsequently 'Total powder'. This was injected in five groups of fifteen rats each in the following doses: 0·1, 0·5, 2, 10 and 50 mg/rat/day. Aortic constriction was performed 14 days later in nine rats of each group and injections were continued for another 5 days. The blood pressure was then measured, the rats killed and the wet and dry weight of their hearts and endocrine organs determined.

Series II. From the above pilot experiment the dose of 10 mg/rat/day was selected, with the exception of TSH, group (6), of which (owing to insufficient supplies) only 5 mg/rat/day was given.

The following nine groups of rats were used:

(1) Hypophysectomized, and examined 14 days later.

(2–8) Hypophysectomized given replacement therapy by injection:

(2) given hormone-free material;

(3) given a fraction containing luteinizing hormone (LH) + TSH + GH;

(4) given a fraction containing GH alone;

(5) given a fraction containing follicle-stimulating hormone (FSH) alone;

(6) given a fraction containing TSH alone.
(7) given the total powder (the same as in Series I);
(8) given a combination of (3) and (4) called 'combined treatment'. (It would have been preferable to combine GH with TSH alone, but not enough of the latter was available.)
(9) Normal unoperated rats.

In each group treatment was given to sixteen hypophysectomized rats for 14 days. In ten of each group the aorta was then constricted with a silver ring of 0·8 mm diameter and treatment was continued for another 8–10 days. The right heart and right femoral artery were then cannulated for sampling and the cardiac output was determined according to the direct Fick principle, with the micro-oxygen determination of Roughton & Scholander (1943). To determine the extent to which cardiac output could be raised, polyvinyl-pyrrolidone (PVP) was infused into the right heart with a Palmer slow-infusion pump. Details of the procedure have been given already (Beznák 1958). Blood pressure was measured in a carotid artery with Hathaway gauges and was recorded photographically, and the heart rate was calculated from these records. From the estimated cardiac output and blood pressure, total peripheral vascular resistance (TPR) was calculated as well as the work of the heart. In the latter case only the work of the left ventricle was considered and the kinetic factor was omitted. 'Basic work' refers to the work done by the left ventricle before the infusion was started, and 'maximum work' to the maximum that could be reached during PVP infusion. Cardiac output is expressed similarly as 'Basic' or 'Maximum' output. Strength of the heart refers to the maximum work done during PVP infusion. Oxygen consumption was measured with a microspirometer and respiration was recorded with a Hathaway gauge. Blood volume was determined by the Evans Blue method, microhaematocrit with the capillary method and the International Microhaematocrit Centrifuge. Erythrocytes were counted and all rats submitted to autopsy after termination of the experiment. At this time the wet and dry weight of the total heart and of its different parts (Beznák 1952*a*), of the endocrine glands and body organs were determined. Only those rats were included in which hypophysectomy was found from macroscopic examination of the sella to be complete. Changes in the weight of the heart and of its different parts were expressed in the following way. Since the weight of the heart very closely follows changes in body weight (Walter & Addis, 1939; Beznák, 1954*b*), cardiac weight had to be related to the final body weight of the rat. The ratio heart weight:body weight was found to be greater in small rats than in large; thus the regression of cardiac, left and right ventricular weight on body weight was calculated by the method of least squares (Beznák, 1954*b*, 1955*b*). In Tables 1*a* and *b* 'Found weight' expresses the actually measured weight 'Calculated weight' the weight expected in a normal rat of the same body weight calculated from the formulae given by Beznák (1955*b*) for Wistar strain rats. The last line of Table 1*a* shows how well the actually measured heart weights of the eight normal rats of this series compared with those calculated from the formulae. 'Difference, in milligrams', then gives the difference between the found and the calculated values. In the groups with aortic constriction (Table 1*b*) comparison was made with their own control groups, i.e. those receiving the same treatment without aortic constriction. The weights of the left and right ventricles were calculated as described by Beznák (1952*a*). Student's *t* test was used for the statistical evaluation.

RESULTS

Series I

There was a direct correlation between body growth and dose administered. With a dose of 10 or 50 mg/rat/day, hypophysectomized rats grew with about the same speed as normal rats. The weight of the adrenals and

thyroids (per 100 g body weight) was about normal in hypophysectomized rats treated with 10 or 50 mg. The weight of the testes and epidydymis was near or above normal with 0·1, 0·5 and 5 mg, but below normal with 10 and 50 mg. The weight of the organs (per 100 g body weight) was normal with 10 and with 50 mg. The atrophy of the heart which occurs after hypophysectomy was practically absent in hypophysectomized rats treated with 2–50 mg total powder/rat/day. Aortic constriction produced a left ventricular hypertrophy of 33 % in normal rats, but not in the hypophysectomized. In hypophysectomized rats treated with 10 or 50 mg total powder, aortic constriction caused a left ventricular hypertrophy of 23 and 25 % respectively. The blood pressure of hypophysectomized rats treated with these large doses of total powder was nearly normal, and hypertension developed after aortic constriction, though it did not reach the level found in normal rats. From these results, 10 mg/rat/day was selected as a suitable dose and was used in Series II.

Series II

Figure 1 shows the body growth of the different groups. All rats lost weight during the first few days after hypophysectomy, but the groups receiving the total powder, LH + TSH + GH, GH alone, or the combined treatment subsequently started to grow and their growth rate was comparable to that of normal rats.

Table 1 shows the weights of the whole heart, the left and right ventricles and their changes. The loss in cardiac weight following hypophysectomy was practically absent in the groups receiving the total extract or TSH. Aortic constriction caused no significant change in the weight of the heart of hypophysectomized rats, but the weight of the heart of normal rats increased by 176 mg. Most of this increase was due to a left ventricular hypertrophy. A comparable increase in the weight of the heart (left ventricle) followed aortic constriction in hypophysectomized groups receiving the total powder or the combined treatment. A smaller but still significant left ventricular hypertrophy occurred in the groups treated with LH + TSH + GH or GH alone. The change of cardiac weight in the other groups was not significantly different from that of the hypophysectomized untreated controls. A comparison of these results with Fig. 1 shows that cardiac hypertrophy followed aortic constriction in those hypophysectomized groups that also grew well, i.e. which received a preparation containing growth hormone. Cardiac hypertrophy, however, was more pronounced in the groups receiving TSH in addition to growth hormone, but TSH alone had very little effect. On the other hand, prevention of cardiac atrophy after hypophysectomy did not depend on growth hormone. Atrophy was absent from the group treated with TSH, although no body

growth took place. Growth hormone, sufficient for good body growth, did not prevent atrophy.

Table 2 shows that the oxygen consumption of hypophysectomized rats was low and that treatment with the total powder or with TSH raised it practically to normal. Comparing these results with those of Table 1,

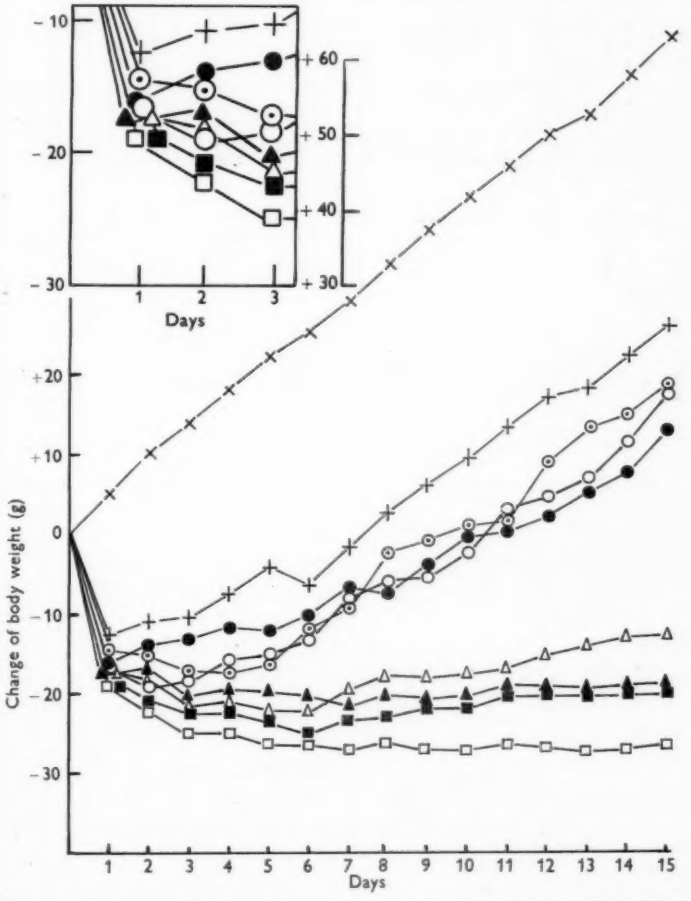

Fig. 1. ×—×, normal rats; +—+, hypophysectomized rats receiving 'Total Powder'; ⊙—⊙, hypophysectomized rats receiving the 'Combined treatment'; ○—○, hypophysectomized rats receiving growth hormone; ●—●, hypophysectomized rats receiving (LH+TSH+GH); △—△, hypophysectomized rats receiving TSH; ▲—▲, hypophysectomized rats receiving hormone-free material; ■—■, hypophysectomized rats receiving FSH; □—□, hypophysectomized rats otherwise untreated. Inset, first three days on larger scale.

TABLE 1. Found and calculated weight (mg) of right and left ventricles and of the whole heart and the difference between the two values

Groups	No.	Right ventricle			Left ventricle			Whole heart		
		Found	Calculated	Difference	Found	Calculated	Difference	Found	Calculated	Difference
				a. Without aortic constriction						
1. Hypophysectomy	9	118±24	168±11	−50±19	354±55	420±23	−66±38	511±78	644±36	−133±62
2. Hormone-free material	9	117±16	159±14	−42±20	337±34	398±31	−61±38	489±47	611±47	−122±54
3. LH+FSH+GH	7	180±51	209±20	−29±60	473±91	505±43	−32±38	697±107	776±66	− 79±76
4. GH	5	145±20	190± 8	−45±23	388±18	465±17	−76±18	573± 32	713±26	−140±34
5. FSH	6	113±17	162±14	−49±12	331±46	404±30	−72±24	484± 61	621±46	−137±31
6. TSH	7	165±24	177±10	−12±16*	416±36	438±20	−22±22*	630± 63	672±31	− 42±43*
7. 'Total Powder'	6	172±16	200±14	−28±18*	465±51	486±30	−21±34*	690± 57	745±45	− 56±44*
8. Combined treatment	8	168±29	204±13	−36±22	439±70	494±28	−55±43	660±108	759±43	− 99±68
9. Normal	8	183±28	180±17	+ 3±18*	442±39	443±34	− 1± 9*	677± 60	679±53	− 2±29*
				b. With aortic constriction						
1. Hypophysectomy	11	132±14	127± 9	5± 9	361±59	330±18	31±48	537±72	493±25	44±53
2. Hormone-free material	9	125±14	113± 7	12±17	376±35	327±17	49±28	544±41	474±25	70±40
3. LH+FSH+GH	12	152±17	147±16	5±12	483±45	398±37	85±36*	690±64	573±56	117±45*
4. GH	11	135±16	127± 6	8±18	436±30	348±12	88±25*	620±43	512±23	108±40*
5. FSH	11	116± 6	97±11	19± 6	346±43	292±11	54±37	502±46	427±17	75±47
6. TSH	9	131±21	130± 8	1±23	397±48	339±19	58±37	572±57	506±28	66±48
7. 'Total Powder'	7	173±18	160±11	13±15	590±73	435±26	155±65*	827±93	647±35	180±75*
8. Combined treatment	9	169±22	155± 9	14±26	549±57	408±21	141±58*	795±67	623±32	172±73*
9. Normal	5	193±30	185±10	8±30	603±79	454±22	149±68*	872±103	696±34	176±88*

Throughout the Tables the nine groups correspond. *All* rats, except those in groups 9 (Normal) were subjected to hypophysectomy, other treatment being additional. The mean ± the standard deviation of the individual cases is given. The weights are calculated from the following formulae: for the right ventricle, $y = 0{\cdot}62x + 62{\cdot}59$; for the left ventricle $y = 1{\cdot}79x + 96{\cdot}41$; for the whole heart $y = 2{\cdot}59x + 186{\cdot}24$; where y = expected weight in milligrams, x = body weight in grams (Beznák, 1955*b*). * Significant difference ($P < 0{\cdot}05$) between this group and control group (1).

it is obvious that atrophy of the heart was greatly diminished in just these two groups. That TSH was responsible for the increase in oxygen consumption is further corroborated by the second part of the Table showing hypophysectomized rats with aortic constriction. A significant increase in oxygen consumption occurred in all groups treated with a preparation containing TSH. No connexion, however, existed between oxygen consumption and left ventricular hypertrophy. Respiration rate was 99 ± 15/min in normal rats and 66 ± 11 in the hypophysectomized. The changes caused by the various treatments paralleled those in oxygen consumption, consequently a detailed tabulation was omitted.

TABLE 2. Oxygen consumption (ml./min/m² body surface)

Groups	Without aortic constriction			With aortic constriction		
	No.		*P*	No.		*P*
1. Hypophysectomy	9	68 ± 10	—	5	73 ± 12	—
2. +Hormone-free material	4	83 ± 20	—	7	80 ± 11	—
3. +LH+TSH+GH	6	$81 + 25$	—	7	105 ± 20	0·01
4. +GH	5	93 ± 37	—	5	86 ± 22	—
5. +FSH	6	93 ± 38	—	9	99 ± 30	—
6. +TSH	6	121 ± 17	< 0·001	7	131 ± 30	0·01–0·001
7. +'Total Powder'	6	122 ± 8	< 0·001	6	121 ± 28	0·01–0·001
8. +'Combined treatment	7	102 ± 23	0·01–0·001	8	95 ± 20	0·05
9. Normal	8	148 ± 22	< 0·001	5	147 ± 23	< 0·001

P = between hypophysectomized control and groups receiving additional treatment.

Table 3 shows that the heart rate was also influenced by TSH. The very pronounced bradycardia of hypophysectomized rats was lessened to varying extent by all fractions containing TSH.

Table 4 shows the cardiac index (output, l./min/m² body surface). Body surface was calculated by Diack's formula (1930). None of the treatments, not even those which practically prevented atrophy of the heart, restored the lower output of hypophysectomized rats to normal. The output, however, was raised significantly in the groups receiving TSH, the total powder or the combined treatment. Cardiac output rose considerably during PVP infusion. The maximum output of normal hearts was three times the pre-infusion value. As was shown by Beznák (1959) the hearts of hypophysectomized but otherwise untreated rats could also raise their output during infusion. The highest outputs were observed in the groups which received TSH, irrespective of whether aortic constriction was present or absent.

The systolic pressures, measured in the carotid artery, are shown in Table 5. The hypotension of hypophysectomized rats was unchanged in groups 2 and 5 and the rise was not significant in group 4. In all other groups the blood pressure was raised to or near the normal level. The same

preparations, plus the one containing growth hormone, accounted for the development of hypertension after aortic constriction. In these groups cardiac hypertrophy also developed, except in the group treated with TSH, where the increase in the weight of the left ventricle was not significant, though hypertension was present.

Higher peripheral vascular resistance (Table 6) accounted for the rise of blood pressure towards normal produced by some preparations despite a subnormal cardiac output. Peripheral resistance was higher in hypo-

TABLE 3. Heart rate (beats/min)

Groups	Without aortic constriction			With aortic constriction		
	No.		P	No.		P
1. Hypophysectomy	9	327 ± 37	—	5	306 ± 54	—
2. + Hormone-free material	4	311 ± 33	—	7	311 ± 36	—
3. + LH + TSH + GH	7	379 ± 50	0·05–0·02	12	386 ± 38	0·01–0·001
4. + GH	5	351 ± 38	—	10	359 ± 63	—
5. + FSH	7	332 ± 49	—	10	369 ± 74	—
6. + TSH	6	405 ± 57	0·01–0·001	9	410 ± 56	0·01–0·001
7. + 'Total Powder'	6	440 ± 40	0·001	6	380 ± 32	0·02
8. + Combined treatment	7	399 ± 48	0·01–0·001	8	368 ± 69	—
9. Normal	8	476 ± 46	0·001	5	444 ± 46	0·001

P = between hypophysectomized and additionally treated groups.

TABLE 4. Cardiac output (l./min/m² body surface)

Groups	Basic output			Maximum output		
	No.		P	No.		P
			Without aortic constriction			
1. Hypophysectomy	9	0·82 ± 0·25	—	9	4·25 ± 0·96	—
2. + Hormone-free material	4	1·17 ± 0·37	—	4	3·85 ± 2·15	—
3. + LH + TSH + GH	6	1·12 ± 0·35	—	6	2·55 ± 0·75	—
4. + GH	5	0·94 ± 0·28	—	5	4·78 ± 2·95	—
5. + FSH	5	0·83 ± 0·26	—	5	2·89 ± 1·04	—
6. + TSH	6	1·26 ± 0·29	0·01–0·001	6	5·60 ± 2·58	—
7. + 'Total Powder'	6	1·28 ± 0·47	0·05–0·02	6	7·08 ± 3·22	—
8. + Combined treatment	7	1·28 ± 0·40	0·02–0·01	7	5·43 ± 2·00	—
9. Normal	8	1·92 ± 0·35	< 0·001	8	5·76 ± 1·71	0·05
			With aortic constriction			
1. Hypophysectomy	5	1·03 ± 0·24	—	5	3·34 ± 0·89	—
2. + Hormone-free material	7	0·82 ± 0·20	—	7	3·39 ± 0·94	—
3. + LH + TSH + GH	6	1·16 ± 0·40	—	6	4·89 ± 2·40	—
4. + GH	5	0·94 ± 0·31	—	5	3·69 ± 0·91	—
5. + FSH	9	1·19 ± 0·57	—	8	3·69 ± 1·13	—
6. + TSH	7	1·37 ± 0·50	—	5	6·80 ± 3·03	0·05
7. + 'Total Powder'	6	1·46 ± 0·46	—	6	5·09 ± 0·94	0·02–0·01
8. + Combined treatment	8	1·10 ± 0·27	—	8	4·23 ± 1·93	—
9. Normal	5	1·59 ± 0·19	0·01–0·001	5	6·14 ± 0·89	< 0·001

P = between hypophysectomized and additionally treated groups.

physectomized than in normal rats and remained high with all the preparations used here.

Cardiac work is expressed in Table 7 in kg.m/min/g left ventricle. The work done by the left ventricles of hypophysectomized rats was much less than that of normal rats (0·061 against 0·158). It was raised by those preparations which contained TSH, which also raised cardiac output and blood pressure. The work of the heart during PVP infusion was greatest

TABLE 5. Systolic pressure in the carotid artery (mm Hg)

Groups	Without aortic constriction: No.		P_1	With aortic constriction: No.		P_2	P_3
1. Hypophysectomy	9	94±24	—	5	102±29	—	—
2. +Hormone-free material	4	86±18	—	7	120±41	—	—
3. +LH+TSH+GH	7	126±27	0·02	12	172±34	0·01–0·001	0·001
4. +GH	5	114±20	—	10	155±26	0·01	0·01–0·001
5. +FSH	7	99±20	—	10	123±22	0·05	—
6. +TSH	6	128±18	0·01	9	161±34	0·05	0·01–0·001
7. +'Total Powder'	6	139±15	0·001	6	175±29	0·02	< 0·001
8. +Combined treatment	7	139±17	0·001	8	161±26	0·05	0·01–0·001
9. Normal	8	136±21	0·001	5	171±14	0·01	< 0·001

P_1 = between hypophysectomized and additionally treated groups; P_2 = between the same group before and after aortic constriction; P_3 = between hypophysectomized groups with aortic constriction and additionally treated group.

TABLE 6. Peripheral resistance (1000 dyn. sec.cm^{-5})

Groups	Without aortic constriction: No.	
1. Hypophysectomy	9	318± 63
2. +Hormone-free material	4	342±162
3. +LH+TSH+GH	7	323±160
4. +GH	5	341±143
5. +FSH	7	400±162
6. +TSH	6	333± 93
7. +'Total Powder'	6	274± 62
8. +Combined treatment	7	318± 78
9. Normal	8	218± 53

P between hypophysectomized and normal group = 0·001

in the groups treated with TSH or with the total powder. The work done by the heart of hypophysectomized rats after aortic constriction was also significantly less than that done by the hypertrophied hearts of normal rats. The average basic work performance of the groups receiving a fraction containing TSH was very near the normal level, but owing to the great scatter the difference was not significant when compared with the value found in untreated hypophysectomized rats. The maximum work done

during PVP infusion, however, was significantly higher in all groups receiving TSH than in the untreated hypophysectomized group; only the hormone-free material and FSH had no effect.

TABLE 7. Cardiac work (kg.m/min/g left ventricle)

Groups	No.	Basic work	*P*	Maximum work	*P*
			Without aortic constriction		
1. Hypophysectomy	9	0·061 ± 0·042	—	0·254 ± 0·123	—
2. + Hormone-free material	4	0·090 ± 0·040	—	0·257 ± 0·110	—
3. + LH + TSH + GH	6	0·108 ± 0·068	—	0·234 ± 0·181	—
4. + GH	5	0·087 ± 0·035	—	0·340 ± 0·210	—
5. + FSH	5	0·068 ± 0·029	—	0·251 ± 0·096	—
6. + TSH	6	0·101 ± 0·040	0·1–0·05	0·463 ± 0·218	0·05
7. + 'Total Powder'	6	0·125 ± 0·055	0·02	0·419 ± 0·097	0·05–0·02
8. + Combined treatment	7	0·130 ± 0·073	0·05–0·02	0·366 ± 0·122	—
9. Normal	8	0·158 ± 0·041	< 0·001	0·361 ± 0·135	—
			With aortic constriction		
1. Hypophysectomy	5	0·066 ± 0·027	—	0·207 ± 0·067	—
2. + Hormone-free material	7	0·059 ± 0·025	—	0·255 ± 0·042	—
3. + LH + TSH + GH	6	0·108 ± 0·057	—	0·385 ± 0·111	0·02–0·01
4. + GH	5	0·085 ± 0·032	—	0·367 ± 0·093	0·01
5. + FSH	9	0·101 ± 0·048	—	0·276 ± 0·091	—
6. + TSH	7	0·118 ± 0·073	—	0·519 ± 0·267	0·05–0·02
7. + 'Total Powder'	6	0·116 ± 0·070	—	0·336 ± 0·100	0·05–0·02
8. + Combined treatment	8	0·089 ± 0·034	—	0·275 ± 0·127	—
9. Normal	5	0·124 ± 0·017	< 0·001	0·360 ± 0·087	0·02–0·01

P = between hypophysectomized and additionally treated group.

No determination of the ACTH content of the fractions was available. Figure 2 shows that some of them caused a considerable increase of adrenal weight in hypophysectomized rats. There was, however, no correlation between increase in adrenal weight and haemodynamic activity of the various fractions. This is in agreement with earlier findings (Beznák, 1952*b*, 1954*c*). Later, unpublished results showed that treatment of hypophysectomized rats with long-acting ACTH preparations ('Adrenomone,' Armour, with gelatin and 'Duractin', Nordisk, with carboxymethylcellulose as vehicle), even in doses which caused pronounced adrenal hypertrophies, had no effect on the cardiovascular parameters discussed here.

Blood volume and red cell count were unchanged 2 weeks after hypophysectomy and none of the treatments had any influence on either.

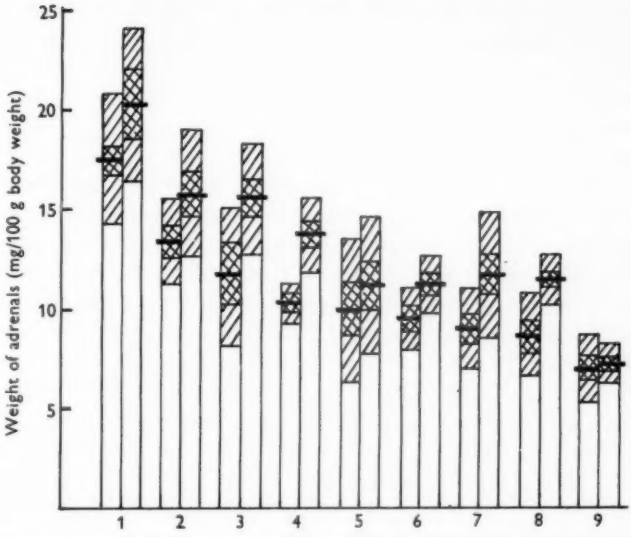

Fig. 2. Weight of the adrenals. 1, normal rats; 2, hypophysectomized rats receiving the 'Combined treatment'; 3, hypophysectomized rats receiving FSH; 4, hypophysectomized rats receiving growth hormone; 5, hypophysectomized rats receiving the hormone-free material; 6, hypophysectomized rats receiving the 'Total Powder'; 7, hypophysectomized rats receiving TSH; 8, hypophysectomized rats receiving LH; 9, hypophysectomized rats otherwise untreated. The first column of each group shows the rats without aortic constriction and the second column those with aortic constriction; cross-hatched area = standard deviation of the individual cases; doubly cross-hatched area = standard error of the mean.

DISCUSSION

The results indicate that TSH is able to prevent the appearance of most of the haemodynamic changes of hypophysectomized rats: Metabolism does not decrease as shown by the nearly normal oxygen consumption. The heart can meet the need of the organism: its output is much less diminished, there is no bradycardia and the blood pressure remains near the normal level. No atrophy of the heart develops. Administration of growth hormone is thus not necessary for the prevention of cardiac atrophy; in fact cardiac atrophy was uninfluenced in the group receiving only growth hormone. These results are compatible with the hypothesis that the haemodynamic changes after hypophysectomy are the consequence of the decreased metabolism, and can be prevented by keeping metabolism at the normal level by the administration of TSH.

Cardiac hypertrophy, on the other hand, followed aortic constriction only in those hypophysectomized rats which were treated with a fraction

containing growth hormone. The hypertrophy was greater in those groups that received both growth hormone and TSH, but TSH alone had very little effect. Similarly Selye *et al.* (1945) showed that the cardiac hypertrophy produced by anterior lobe and STH preparations in normal rats was appreciably accentuated by combination with thyroid hormone treatment. The experiments of Salgado (1955, 1957) also indicated that both growth hormones and TSH were needed to produce hypertension and cardiac hypertrophy in hypophysectomized rats. This could indicate either that TSH increased the response of the cells to growth hormone or that by its effect on metabolism it added a further strain on the heart already loaded, e.g. by the constriction of the aorta, and that this additional strain, in the presence of growth hormone though not without it, led to a greater enlargement of the heart. Experimental evidence is in favour of the former possibility. Other effects of growth hormone, e.g. body growth, are also influenced by the thyroid. Thus Evans, Simpson & Pencharz (1939), Salmon (1941), and Laqueur, Dingemanse & Freud (1941), found that the effect of STH was absent or diminished in the absence of the thyroid. Treating hypophysectomized rats with different growth hormone preparations, Beznák (1956) found that cardiac hypertrophy followed aortic constriction if the growth hormone preparation was not highly purified, but with highly purified preparations no cardiac hypertrophy resulted. This indicates that someting else may have to be present for growth hormone to have any action at all. The two processes, the prevention of cardiac atrophy and the development of hypertrophy, are thus shown to be different. Cardiac atrophy in hypophysectomized rats is a secondary process, and TSH, by removing its cause, the decreased metabolism, prevents its appearance. Cardiac hypertrophy in consequence of a permanently increased load on the heart, is an anabolic process, entailing formation and deposition of new material, and for this growth hormone is necessary, though TSH potentiates its effect.

Regarding the connexion between size and work of the heart, if they were strictly interlinked processes, the work done per gram of heart muscle should not vary. The results indicate that this is not the case. The heart becomes not only smaller after hypophysectomy but it also does less work per gram than the hearts of normal rats. If the normal heart is confronted with a constant load, hypertrophy develops. Though the actual total work done by the hypertrophied heart is greater than in the normal heart, there is a tendency for the work per gram to diminish. This is less so with the medium hypertrophies of normal heart muscle. Thus Bretschneider, Bücherl, Frank & Husten (1952) found cardiac work per gram, and efficiency, the same in heart–lung preparations of normal and hypertrophied dog hearts. Though the work of the heart was not measured,

Beznák (1954*a*) found no correlation between left ventricular weight and blood pressure. If the aorta was markedly constricted, the weight of the heart increased sharply while hypertension diminished (Beznák 1955*a*).

The strength of the heart is measured here by the maximum work it can do when suddenly confronted with an increased load produced by PVP infusion into the right heart. The results show that the highest work performance was reached in the group receiving TSH. This is especially noteworthy after aortic constriction, because no cardiac hypertrophy developed in this group. On the other hand, in the growth-hormone-treated group the left ventricles were enlarged, yet the maximum work done was less than in the TSH-treated group. It is concluded from these results that growth hormone controls the mass changes and TSH the work- and force-producing ability of the heart.

All the effects of the preparations used in these experiments can thus be explained by their containing GH, TSH or both. Some of them, however, also contained LH and FSH. Though Korenshevsky, Hall & Ross (1939) could produce some cardiac hypertrophy in rats by male-sex-hormone treatment and noted a slight diminution of the heart in castrated rats, it seems unlikely that the sex hormones play any major part in these experiments. Most of the preparations used also increased the weight of the adrenals of hypophysectomized rats, but there was no correlation between adrenal size and cardiovascular effectiveness. Rather (1954) showed that renal hypertension and cardiac hypertrophy diminished after adrenalectomy in rats. Selye & Bois (1955) found that adrenalectomy prevented the nephrotoxic and vasotoxic action of STH. Hoelscher (1954) determined the cardiac output of adrenalectomized rats and found it below normal. It seems therefore probable that some adrenal active material must be present to assure normal responsiveness to other hormones. The adrenal function remaining after hypophysectomy seems, however, sufficient for the other hormones to exert their cardiovascular effects.

SUMMARY

1. Hypophysectomized rats (with and without aortic constriction) were treated with a total sheep pituitary powder, and with five fractions prepared from it. To establish the cardiovascular actions of these different fractions the following parameters were measured: cardiac output and work, strength of the heart (measured by the maximum to which output and work could be raised during PVP infusion into the right heart), heart rate, blood pressure, total peripheral resistance, oxygen consumption and respiration, blood volume, microhaematocrit and red cell count, size of the total heart and of its different parts.

2. The haemodynamic changes after hypophysectomy were found to be secondary to the decreased metabolism, and TSH by keeping metabolism at the normal level prevented their appearance; no bradycardia developed, cardiac output and work decreased less, there was no atrophy of the heart and the blood pressure did not fall.

3. Constriction of the aorta in hypophysectomized rats led to cardiac hypertrophy only in those groups which were treated with a fraction containing growth hormone. The hypertrophy was greater if the rats received TSH as well as growth hormone, though no significant cardiac enlargement followed aortic constriction in hypophysectomized rats treated with TSH alone. The two processes, prevention of cardiac atrophy and the development of cardiac hypertrophy, are thus different, the former being controlled by TSH, the latter by growth hormone.

4. Size, work and strength of the heart were found to be not strictly interlinked processes. After hypophysectomy the heart became smaller but also did less work per gram of muscle than normal hearts (0·061 against 0·158 kg.m/min/g left ventricle) and there was a tendency for hypertrophic hearts to do less work per gram. The strength of the heart, i.e. its maximum work-producing ability, was greatest in the group receiving TSH, although in this group no cardiac hypertrophy followed aortic constriction. It is concluded that size and strength of the heart can be influenced separately, the former by growth hormone, the latter by TSH.

The work was supported by a National Health Grant, Canada, given through the Ontario Heart Foundation. I wish to express my gratitude to the Endocrinology Study Section of the National Health Institutes, Bethesda, U.S.A., to Drs Wilhelmi and Ellis for the generous supply of the material used, to the Nordisk Laboratories for a gift of Duracton and to Mrs T. Fournier and Mr L. Roman for their excellent technical assistance. The material was supplied through the Endocrinology Study Section of the National Health Institutes, Bethesda, Md., U.S.A. The fractions were prepared by Dr Ellis in A. E. Wilhelmi's Biochemical Department, Emory University.

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THE ROLE OF NON-MYELINATED FIBRES IN SIGNALLING COOLING OF THE SKIN

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Comparatively little is known of the sensory functions of the non-myelinated fibres which make up the bulk of the afferent population in cutaneous nerves. Although it is widely held that these C fibres are 'pain fibres' and signal noxious stimuli, there is recent evidence indicating that they play an important part in signalling stimuli which are clearly not painful, such as touch and light pressure (Douglas & Ritchie, 1957*b*; Iggo, 1958). The present experiments were done to learn more of the sensory functions of the non-myelinated afferent fibres in cutaneous nerves by studying their electrical activity and examining whether or not they signal changes in skin temperature.

Cooling has so far been recognized to be signalled by small myelinated fibres of the A delta type and the behaviour of the peripheral sensory apparatus in response to temperature changes is commonly discussed in terms of such specific 'temperature fibres' (Zotterman, 1936, 1953; Hensel & Zotterman, 1951*a*, *b*; Maruhashi, Mizuguchi & Tasaki, 1952; Boman, 1958). But it appeared from preliminary studies (Douglas, Ritchie & Straub, 1959) that in a typical cutaneous nerve cold is signalled not only by delta fibres but also by a large body of C fibres. These C fibres, like the delta 'cold fibres', responded to moderate cooling of the skin and fired when the skin temperature was lowered by amounts unlikely to cause pain.

Since such fibres seemed to make up a large fraction of the whole population of C fibres in the cat's saphenous nerve and since histological studies (Gasser, 1950) have shown that non-myelinated fibres are about three or four times as numerous as all myelinated fibres in this nerve, it appeared worth while to examine their response to cold in more detail.

METHODS

The experiments were carried out on cats anaesthetized with chloralose (80–100 mg/kg body weight) given intravenously after induction with ethyl chloride and ether.

Preparation. The hair on the right hind limb was cut short with electric clippers and the cat was placed on its back with the right leg held everted in extension by a clamp on the

foot. The skin over the saphenous nerve was incised from the groin to about midway between the knee and the ankle, reflected from the muscle and held by threads to form a bath. This bath was filled with liquid paraffin at about 30–35° C which had been equilibrated with a gas mixture composed of 5% CO_2 in O_2, and which had previously stood over Locke's solution (mM: NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 1·9; dextrose, 5): unless wetted in this way some liquid paraffins cause the nerve to dry. A branch of the saphenous nerve usually leaves the main trunk just below the knee and runs with a small artery and vein to supply an area of several square centimetres of skin over the lateral aspect of the leg from the ankle to the knee. This knee branch was freed from the connective tissue to the point where it joined the main saphenous trunk. The saphenous trunk below this point was cut and the saphenous nerve above it separated from the underlying connective tissue as far up as the inguinal ligament, where it was cut.

Cooling. The arrangement for cooling the skin is shown in Fig. 1. A bath of thin metal foil was placed against the skin and thermal stimulation achieved by replacing the water in the bath by water at different temperatures. It took about 2–3 sec to empty the bath and less than a second to fill it. Since the skin supplied by the knee branch of the saphenous nerve has an area of several square centimetres and varies somewhat in position and shape in different animals, the bath was made of thin aluminium foil so that it could be moulded to the particular shape of this area of skin in each experiment. The bath was held in close contact with the skin by a scaffolding of wood, 'Plasticine' and wire so that filling or emptying it caused little mechanical movement. The temperature of the water in the bath was measured with a rapidly responding thermocouple and galvanometer system. Another such thermocouple was sandwiched between the bath and the area of skin supplied by the knee branch, to give an estimate of the superficial skin temperature.

Stimulating and recording. Sensory discharges in specific groups of nerve fibres were measured by observing the modulation of the corresponding components of an antidromic compound action potential occurring as a result of occlusion. The principle will be discussed later (pp. 268–9). Bright platinum electrodes were used and their arrangement on the nerve is shown in Fig. 1. One pair of electrodes (R) was placed on the knee branch of the saphenous nerve and used for recording. A single electrode on the main saphenous trunk or on the adjacent muscle acted as an earth. Two pairs of electrodes (S_1 and S_2) were used for stimulating the saphenous nerve trunk high in the thigh. An electrical shock whose intensity was large enough to excite only the A fibres was applied at S_1: a stronger shock exciting C fibres as well as the A fibres was applied at S_2. Pairs of such shocks were usually applied at regular intervals two, three or four times every 2 sec. Each shock at S_1 preceded that at S_2 by about 30 msec. This procedure was adopted for two reasons: first, to obtain records of A potentials free from the artifact which is usually introduced by the intense stimuli necessary to excite C fibres; and secondly, to avoid the deterioration of A fibres which occurs at stimulating electrodes passing the large currents necessary to excite C fibres. The electrical stimuli were rectangular pulses of current applied through radio-frequency isolation units (Schmitt, 1948).

The compound action potentials set up at S_1 or S_2 were led off at R and fed through a cathode follower into two separate amplifiers. Each amplifier was resistance-capacity coupled and had a low-frequency response sufficiently good not to distort the slowest potentials in which we were interested. One amplifier had a high-frequency response such that appreciable falling off in response did not occur until about 20 kc/s: this amplifier was used to record evoked A potentials and also fed a loudspeaker. The other amplifier was used to record the evoked C potentials and had high-frequency shunts to minimize the background of random sensory A fibre activity which otherwise tended to obscure these small evoked C potentials. In this amplifier the high-frequency response was reduced to one half of its maximum height at a frequency of 100 c/s. This did not distort the evoked C potentials which were to be measured to any appreciable extent. The outputs from the two amplifiers

were fed into two adjacent oscilloscopes and the evoked A and C potentials were displayed on different time bases to be photographed together on moving film.

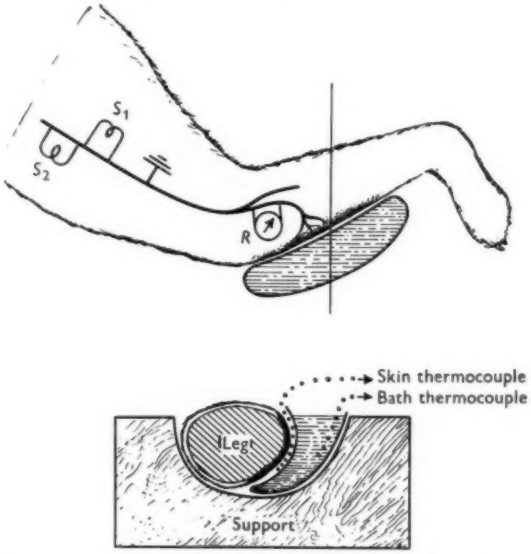

Fig. 1. The arrangement for recording activity in the saphenous nerve and for cooling the skin. The upper diagram of the cat's right hind leg, as seen from above with the cat on its back, shows the positions of the stimulating (S_1 and S_2), recording (R) and earthing electrodes on the saphenous nerve and its knee branch. The area of skin supplied by the knee branch is shown by the stippling. An aluminium foil bath made in the form of a child's paper hat was moulded around this area of skin. The lower diagram is a cross-section through the leg in the plane indicated in the top diagram and shows further the moulding of the bath and the arrangement of the supporting scaffolding. A further support not shown in the diagram was provided by a wire which held the inner wall of the bath closely against the skin. The lower diagram also shows the position of the two thermocouples used to measure bath and skin temperature. The arrangements for filling the bath and emptying it are not shown; it was filled through a wide-bore filter funnel clamped in position above it and emptied through an indwelling suction line.

RESULTS

The technique we have used to detect and measure discharges in non-myelinated fibres (Douglas & Ritchie, 1957*a*) has already been used to show that C fibres in the saphenous nerve respond to touch (Douglas & Ritchie, 1957*b*). It is based on the principle of occlusion. When the saphenous nerve is stimulated electrically high in the thigh (S, Fig. 1), a compound action potential is evoked which propagates antidromically towards recording electrodes (R, Fig. 1) nearer the sensory endings. If

these nerve endings are not discharging, a maximal diphasic antidromic action potential is recorded at *R*. On the other hand, when sensory activity *is* present the amplitudes of particular components of the compound action potential fall because orthodromic impulses in active nerve fibres tend to collide with, and thus extinguish, the evoked antidromic impulses travelling in the same fibres. The reduction in the amplitude of a given elevation of the compound action potential not only indicates which group of fibres is involved, but also gives a measure of the intensity of the discharge. If the individual active fibres are discharging at a frequency sufficiently high to ensure that collision between an antidromic and an orthodromic impulse occurs in all of them, the percentage reduction in a given elevation is equal to the percentage of fibres which are active, for it may be assumed that the amplitude of any given elevation in the compound action potential is proportional to the number of fibres contributing to it. In many cases, however, the frequency of discharge may be lower, so that such occlusion occurs only in some of the active fibres; in such instances the percentage reduction in the corresponding elevation is less than the actual percentage of fibres active. In all cases the percentage reduction in the amplitude of a given elevation gives a lower limit to the number of fibres active; for example, a 30 % reduction means that *at least* 30 % of the corresponding fibres are active. This method we have used is particularly well suited to studying C fibres whose conduction velocity is low, because the reduction of the evoked compound action potential caused by a sensory discharge is inversely proportional to the conduction velocity of the corresponding fibres (Douglas & Ritchie, 1957*a*). For the same reason the method is less well suited to detecting discharges in faster conducting fibres.

The response of myelinated and non-myelinated fibres to cooling the skin

The main finding, that C fibres as well as A delta fibres discharge when the skin is cooled, is illustrated in Fig. 2, which shows antidromic compound action potentials recorded from the knee branch of the saphenous nerve before and during the application of cold to the area of skin it supplies. Three pairs of records are shown. In each pair the upper trace shows the A complex with its two principal components, the alpha-beta and the delta elevations, while the lower trace shows the C complex obtained on a much slower time base. In our experiments where records were made diphasically from the intact nerve this C complex always showed two principal elevations: the faster and larger of these, whose peak propagated at about 1 m/sec at 37° C, will be referred to as the C_1 elevation and the slower, whose peak propagated some 10–30 % more slowly, as the C_2 elevation. The left-hand pair of traces (Fig. 2*a*) are the control potentials obtained when the superficial skin temperature and the bath temperature were

33·2° C. After these control records had been obtained, the bath was emptied and refilled with water at 16·8° C. The middle pair of traces (Fig. 2*b*) was obtained 0·5 sec after applying this cold stimulus, and shows a large reduction in the amplitude of the delta elevation and a very small reduction in that of the alpha-beta elevation. Clearly, occlusion has occurred in both groups of A fibres, particularly in the delta fibres, and it follows that such fibres must have been discharging sensory impulses. There is no reduction in either the C_1 or the C_2 elevations in Fig. 2*b* and it may be concluded that at this time C fibres were little, if at all, excited by the cold stimulus. The right-hand pair of records (Fig. 2*c*) was obtained

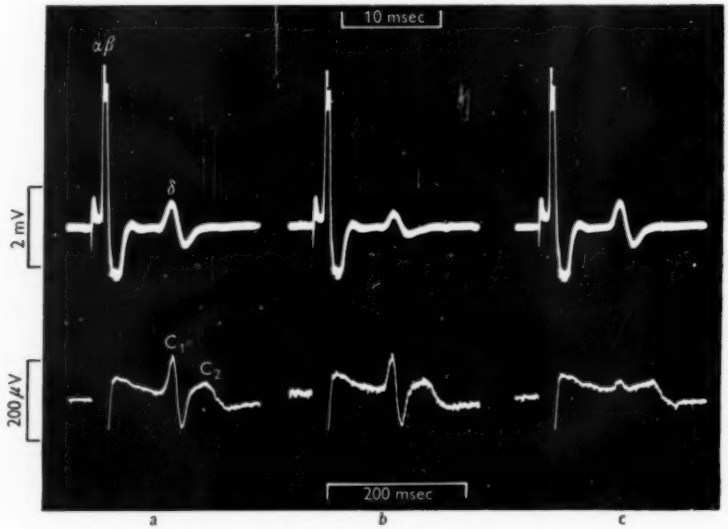

Fig. 2. The activity produced by cooling the skin in the different fibre groups in the cat's saphenous nerve. The upper records show the antidromic compound action potential of the myelinated fibres: there is a large alpha-beta elevation soon after the stimulus artifact, followed some time later by a smaller delta elevation. The lower record, taken with a much slower time base, shows the antidromic compound action potential of the non-myelinated fibres which appears much later. This C complex shows a larger, faster component, the C_1 elevation, and a smaller, slower component, the C_2 elevation. In these lower records the alpha-beta and delta elevations are lost in the artifact. The two left-hand records, *a*, are the controls taken when the skin temperature was at 33·2° C; the middle records, *b*, were taken 0·5 sec after the bath water at 33·2° C had been replaced with water at 16·8° C; the right-hand records, *c*, were taken a further 3·3 sec after *b*, the cold stimulus being maintained. The conduction distance of the A fibres was 66 mm and that of the C fibres 71 mm. The temperature of the nerve trunk was 30° C. Note the fall in both the alpha-beta and delta elevations in record *b* and the fall in the C_1 elevation in record *c*.

3·8 sec after the application of the maintained cold stimulus. By this time the skin temperature had fallen to its lowest value of 22·4° C. The alpha-beta and delta elevations had recovered, but now there was discharge in the bulk of the faster conducting C fibres, for the C_1 elevation is only a small fraction of its control amplitude. The slower C fibres, however, did not seem to respond to the stimulus, for the C_2 elevation is not appreciably reduced.

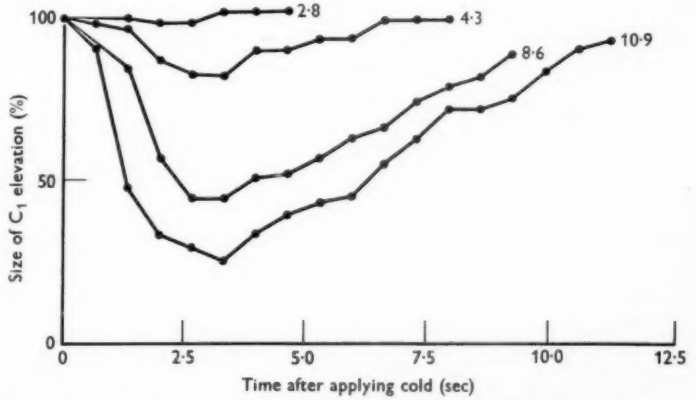

Fig. 3. The response of the C_1 fibres in the cat's saphenous nerve to different intensities of cooling. The ordinate is the size of the C_1 elevation of the compound antidromic action potential and the abscissa is the time after the application of a cold stimulus produced by replacing the bath water at 33° C with water at lower temperatures (at 16·8, 20·5, 26·5 and 29·0° C). The numbers beside each of the four curves indicate the corresponding falls in skin temperature. Recording conditions as in Fig. 2.

The time course of the discharges

The time course of the C_1 fibre discharge set up when the skin is rapidly cooled is shown in Fig. 3. The curves in this figure show the responses of C_1 fibres to cold stimuli of different intensities. Each curve shows the amplitude of the C_1 elevation (expressed as a percentage of its control value) plotted against time after the onset of cooling. It can be seen that even with the coldest stimulus the effect took several seconds to reach a maximum and required many seconds more to pass off. The response of the delta fibres was more transient: it reached a maximum within 1 sec when the C_1 fibre discharge was reaching its maximum. A comparison of the time courses of the effects on the two groups of fibres is shown in Fig. 4: the thick interrupted line shows the beginning of the C_1 fibre response and the thick continuous line the whole of the delta fibre response.

In this experiment the responses of the two groups of fibres were obtained in consecutive tests rather than in the same test. This was done

so that the brief time course of the delta fibre response could be adequately followed by stimulating the A fibres alone at a rate of 10 shocks/sec. This rate is too high for studying the C fibres; these were excited in a subsequent test at 1 shock/sec.

While the responses of the delta and C fibres just described are largely due to cold, some effect was observed when the water in the bath was changed for water at the same temperature and must be ascribed to a discharge set up in mechano-sensitive fibres by the small mechanical movements of the bath occurring during this manœuvre. This mechanical effect was very small in the C_1 group (the thin interrupted line in Fig. 4),

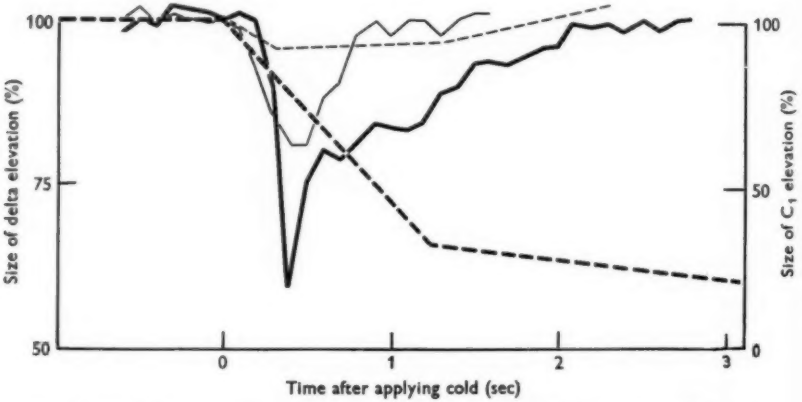

Fig. 4. The different time courses of the delta and C_1 fibre discharges to cooling the skin. The thick continuous line shows the delta fibre discharge produced by lowering the skin temperature from 35 to 24° C: the thick interrupted line shows the C_1 fibre discharge produced by lowering the skin temperature from 33·2 to 25° C. The responses were obtained consecutively from the same nerve. The thin lines show the corresponding discharges set up mechanically by replacing the bath water at about 34° C with water at the same temperature. Conduction distance for delta and C_1 elevations, 71 mm. Temperature of nerve trunk, 32° C.

and was over some seconds before the maximal discharge occurred in response to the cold stimulus: it did not obscure in any way the major portion of the response to cooling. On the other hand, there was a greater mechanical effect in the delta group (the thin continuous line in Fig. 4) and its peak coincided in time with the peak effect seen on cooling. However, the response of the delta fibres to cold could always be detected by the greater effect produced by the cold stimulus.

The relation between temperature change and C_1 fibre activity

The response of the C_1 fibres varied with the extent to which the skin was cooled and was greater the greater the cooling (Fig. 3). In the experiment of Fig. 3 the skin was maintained at about 33° C for several minutes before each test, which consisted of emptying and refilling the bath with water at a lower temperature. When the skin temperature was lowered 10·9° C the C_1 potential fell to about 25 % of its control amplitude, i.e. about 75 % of the C_1 fibres must have fired. This was the maximal effect: lowering the skin temperature by as much as 30° C (i.e. down to about 3° C) did not produce any greater reduction in the C_1 elevation. Falls in skin temperature less than 10·9° C resulted in smaller reductions of the C_1 elevation, a just detectable reduction in the C_1 elevation being obtained with a fall in skin temperature of about 3° C.

When the *fall* in skin temperature lay in the range 3–11° C, there was a simple relationship between the fall in skin temperature and the maximal C_1 fibre discharge (as judged by the maximal reduction of the C_1 elevation) in each test. Thus, when the maximal reductions in the C_1 elevation obtained in the various tests were plotted against the logarithms of the corresponding reductions in skin temperature, the points obtained were fitted by a straight line, as shown in Fig. 5. With falls in skin temperature in excess of about 11° C there was no increased reduction in the C_1 elevation and the curve drawn to fit the points in Fig. 5 becomes flat.

It has already been mentioned that the reduction in a given component of the compound action potential depends not only on the proportion of fibres which are active, but also on their frequency of discharge—the higher the frequency the greater the chance that occlusion will occur. This must be taken into account when the correlation between the observed effect on the C_1 elevation and the applied cold stimulus is considered. For example, although a fall in skin temperature of about 3° C was required to give a just detectable reduction in the C_1 elevation, it does not follow that this represents the threshold cold stimulus for the most sensitive C_1 fibres. It is probable that the fall in skin temperature necessary to excite the most sensitive fibres is less than 3° C. With the method we used it was difficult to detect reductions in amplitude less than about 5 %, so that 5 % of the fibres could have been discharging vigorously without their being detected. Similarly, activity in many fibres, but at low frequency, would escape detection; for example, with a typical interelectrode distance of 75 mm, if *all* the fibres were to fire less than once every 3 sec the reduction in the compound action potential would be less than 5 % (see Douglas & Ritchie, 1957*a*).

Although no further decrease in the amplitude of the C_1 elevation

occurred with falls in skin temperature in excess of about 11° C, it does not follow that colder stimuli did not set up more activity in these C_1 fibres already activated. What the experiment indicates is that the frequency of firing set up by lowering the skin temperature 11° C was so high that occlusion occurred in all the fibres which were cold-sensitive and that these made up about 75 % of the C_1 group. Again, by the calculation given by Douglas & Ritchie (1957*a*) it can be shown that with a typical inter-electrode distance of 75 mm the frequency of discharge of the whole

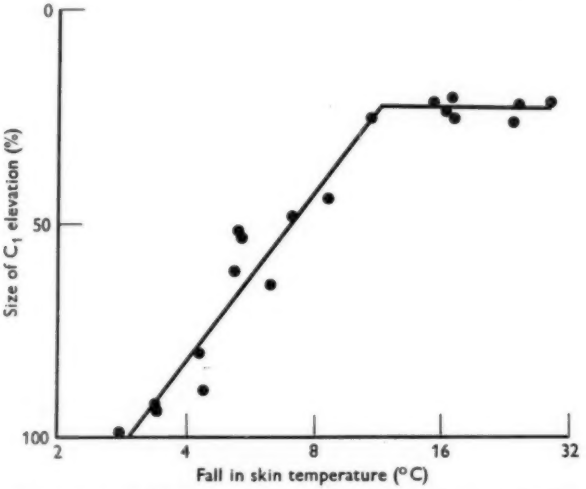

Fig. 5. The relationship between the maximum fall in the C_1 elevation and the change in skin temperature when the skin temperature is lowered by various amounts from a constant initial value (33° C). The temperature change is plotted on a logarithmic scale. Conduction distance, 71 mm; temperature of nerve trunk, 30° C.

population of the cold-sensitive C_1 fibres (conducting at about 1 m/sec) which would just ensure complete occlusion occurring in all of them, and hence a maximal reduction of the C_1 elevation, is about 7 impulses/sec. In these circumstances where complete occlusion was already occurring, an increase in frequency of discharge could clearly cause no greater effect on the C_1 elevation.

The effect of initial temperature on the C_1 fibre response to cooling the skin

The question arises whether or not the response of the C_1 fibres to cooling the skin depends on the initial temperature from which the change in temperature is effected. The responses which have already been shown

(Fig. 5) were produced by cooling the skin in successive tests from a constant initial temperature of about 33° C to various final temperatures. Other tests were done in which the skin was cooled from various initial temperatures below 33° C to a constant final temperature of about 24° C and these gave similar results. As can be seen in Fig. 6, the relationship between the maximum response of the C_1 fibre population and the fall in

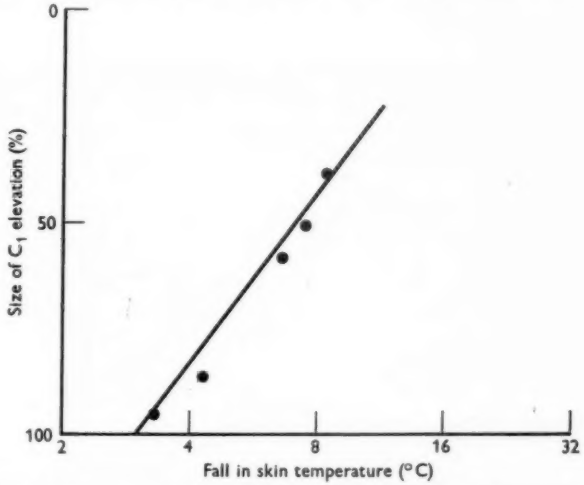

Fig. 6. The relationship between the maximum fall in the C_1 elevation and the change in skin temperature when the skin temperature is lowered from various initial temperatures to a constant final temperature of about 24° C. This experiment was carried out in the same preparation as that used for Fig. 5. Note that although the cooling procedure is different (similar reductions in skin temperature were obtained, but in different temperature ranges), the relationship between the fall in the C_1 elevation and the change in skin temperature is the same, for the line which appears in this figure and which fits the experimental points is the same line which was drawn to fit the experimental points in Fig. 5.

skin temperature is much the same as that found earlier in the same animal (Fig. 5) when the skin was cooled by varying amounts from a constant initial value. It appears, therefore, that over this range of temperature the magnitude of the C_1 fibre response is determined by the *fall* in skin temperature rather than by the initial or final temperatures.

The effect of ethyl chloride

Ethyl chloride sprayed on man's skin produces the sensation of intense cold. Some experiments were done to see whether or not those C fibres in the cat's saphenous nerve which were not excited by cold applied with the bath technique (i.e. all the C_2 and some C_1 fibres) would respond to spraying

with ethyl chloride. When the cat's skin supplied by the knee twig of the saphenous nerve was sprayed for a few seconds with ethyl chloride, there was a vigorous discharge of impulses and the antidromic C_1 elevation was reduced in size (Fig. 7) often to less than 10% of its control amplitude. This is a larger effect than was obtained in the experiments with the bath, even when the skin was cooled to as low as 3° C. This greater effect on the C_1 elevation may have been due to a greater or more rapid fall in skin

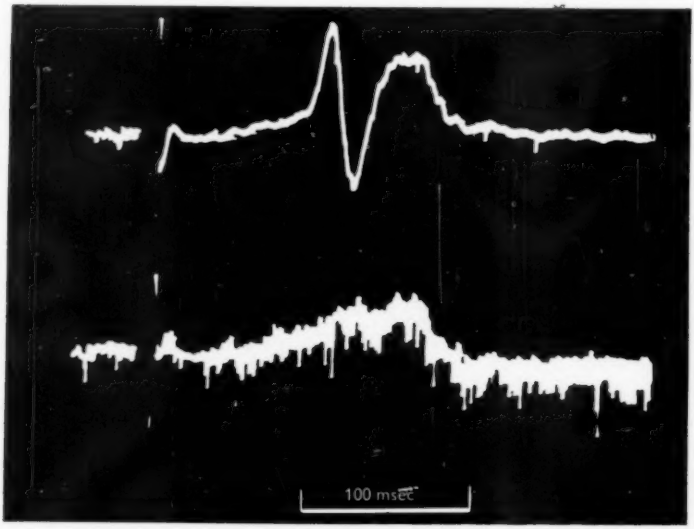

Fig. 7. The sensory discharge in C_1 fibres in the cat's saphenous nerve produced by spraying the skin with ethyl chloride. The upper record was taken before spraying, when the skin temperature was 26·5° C, and the lower record during spraying. The C_1 elevation is greatly reduced in amplitude. There is little change in the C_2 elevation. Conduction distance, 86 mm. Temperature of nerve trunk, 30·5° C.

temperature with ethyl chloride; but it may have been the result of added activity in purely mechano-sensitive C_1 fibres, for spraying the skin with water at normal skin temperatures excited many C_1 fibres, causing the C_1 potential to fall by up to 25%. Spraying with ethyl chloride gave no clear-cut effect on the C_2 fibres; in a few experiments small reductions in the C_2 elevation were noticed but they were too small (about 5%) to be definite.

The effect of heating the skin

In previous experiments (Douglas & Ritchie, 1957*b*) it was found that warming the skin by radiant heat did not produce any detectable discharge

in C fibres. This has been confirmed in the present experiments even when the radiant heat used was so intense as to singe the skin in about 10 sec and was intolerably painful when applied to our own skin. Experiments with the bath technique gave similar results. Thus, when the bath water at about 35° C was replaced with hotter water varying in temperature from 40 to 100° C no reduction in any of the main C elevations was detected.

A common pathway for touch and cold

The results which we have described in the present paper indicate that the majority of C_1 fibres in the cat's saphenous nerve can be excited by cooling the skin. Previous experiments (Douglas & Ritchie, 1957*b*) showed that most of the fibres in this same group are excited by touch. Clearly there must be many C_1 fibres which are sensitive to both forms of stimulation. This can best be demonstrated by applying both stimuli separately

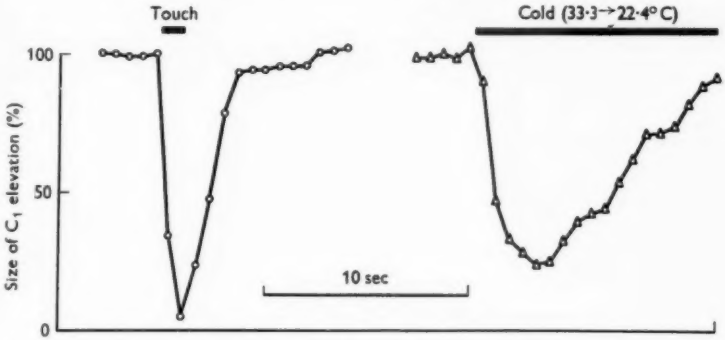

Fig. 8. An experiment showing that C_1 fibres respond both to touch and to cold. Both tests were made on the same nerve. The response to touch was evoked by drawing a roll of cotton gauze across the skin during the period marked by the short horizontal bar. The response to cooling occurred when the skin temperature was lowered 11° C (from 33°) during the period marked by the long horizontal bar.

to the same preparation, as has been done in the experiment of Fig. 8. In the first part of this experiment the skin was stroked with a cotton swab and the C_1 elevation all but disappeared (left-hand record, Fig. 8); in the second part, after the aluminium bath had been mounted and the skin cooled from 33 to 22° C the same C_1 elevation fell by about 75%. Thus touch had excited nearly all the C_1 fibres while cooling had stimulated at least 75% of them. This can only mean that most of the C_1 non-myelinated fibres are sensitive both to touch and to cold.

The influence of electrical stimulation on the sensory discharges

It might be questioned how far the results we have obtained are influenced by the unconventional method of recording sensory activity which has involved regular and prolonged electrical stimulation of the saphenous nerve. The saphenous nerve contains sympathetic motor fibres and there have been numerous reports asserting or denying that activity in the sympathetic efferent nerves influences sensation (see von Brücke, 1932). In a recent study, however, Loewenstein (1956) has clearly demonstrated that stimulation of the sympathetic nerve supply may increase the sensitivity of tactile receptors in the frog's skin. Whatever the effect of concurrent sympathetic stimulation is in our experiments, it could be regarded as a normal physiological rather than an abnormal event, for in stimulating some 2–4 times every 2 sec we were probably mimicking the normal resting sympathetic nervous outflow, which is estimated to be about 1–3 impulses per second (Girling, 1952; Folkow, 1952; Celander & Folkow, 1953; Celander, 1954). The rate of stimulation we have used is certainly well below the maximum frequency of sympathetic discharge, which is about 10 impulses/sec (Pitts & Bronk, 1941; Celander, 1954; Douglas & Ritchie, 1957*a*, *c*).

The method of recording activity we have used, however, involves not only orthodromic stimulation of sympathetic fibres but also antidromic stimulation of all sensory fibres in the nerve. Such antidromic stimulation has been reported to cause not only vasodilatation but also hyperalgesia (Lewis, 1942). This raises the question whether or not the fibres we are studying in the C_1 group are sensitive to moderate cooling or touch only because of the antidromic technique, i.e. whether or not we are studying some abnormal response of 'pain fibres' usually sensitive only to noxious stimuli. The nature of the sensory disturbance observed by Lewis in the skin after antidromic sensory stimulation, however, would make such an interpretation improbable, for in these circumstances the sensory responses to light touch and to cold are not exalted; rather the characteristic change in the skin is that the threshold to various *painful* stimuli is lowered. One such painful stimulus is heat and we have already described how the C_1 fibres do not discharge in response to heat.

In some experiments an attempt was made to examine directly how C_1 fibres respond in the absence of antidromic stimulation. After setting up the nerve on the electrodes and stimulating it only long enough to ensure the adequacy of the stimulating and recording conditions, stimulation was stopped and the animal was left for 1 or 2 hr. At the end of this time the skin was cooled or touched. As soon as the sensory discharge heard in the loudspeaker became intense a single compound antidromic action potential

was sent down the nerve in the usual way to be recorded at the recording electrodes, so that it could be compared with a later control record obtained in the resting preparation. By this means it was possible to obtain a measure of the orthodromic sensory activity during cooling or touching the skin in a preparation where no antidromic impulse had reached the sensory nerve endings within the last 1–2 hr. The amplitude of the C_1 component of this antidromic compound action potential was always smaller than the succeeding control records obtained in the absence of sensory stimulation of the skin. It could thus be inferred that the C_1 fibres were excited by cold or touch even in the absence of recent antidromic activity.

DISCUSSION

Although non-myelinated fibres are thought to be involved in signalling painful stimuli, the degree of cooling which we found excited the fibres in the C_1 group cannot be considered noxious. Some of these cold-sensitive C fibres were excited by a fall of skin temperature of only 3° C and the bulk of them by a fall of about 11° C. Such stimuli applied to our own skin never caused pain. These C fibres are thus capable of signalling quite moderate innocuous cooling of the skin, participating in the afferent discharge from the skin whenever it is cooled. In this sense they may be regarded as 'cold fibres'.

It is interesting to compare the properties of these non-myelinated cold-sensitive fibres with those of the myelinated cold-sensitive fibres of the delta group studied in the present experiments. The general time course of the discharge set up by an instantaneous maintained cooling is much the same in the C_1 fibres as it is in the delta fibres. In both populations the sensory discharge rapidly rises to its maximum and then declines markedly, so that the amplitudes of both the delta and the C elevations soon become indistinguishable from their control values. The C_1 fibre discharge differs from that of the delta fibres principally in that the maximal response is reached a second or two later and declines more slowly. This might provide a mechanism for maintaining a higher degree of sensory discharge during a prolonged cooling than would be possible with delta fibres alone. It would be interesting to know whether these differences between the cold-sensitive fibres of the C_1 and the delta groups reflect a difference in the properties of their endings or some difference in their anatomical location in the skin. Single-fibre experiments on delta fibres in the tongue and in the skin of the face have shown that even though adaptation does take place during a maintained cold stimulus the rate of firing does not recover to normal, but rather to some small fraction of the maximal rate seen soon after the onset of cooling (Hensel & Zotterman, 1951*a*; Boman, 1958). Since this did not show up in our records of the delta elevation, it may be

that a low-frequency discharge (say less than one impulse in each fibre every 3 sec, as described above) also occurs in the C fibres and has similarly escaped detection.

One striking result in our experiments was that over the range of temperatures we studied (24–33° C) the response of the C_1 fibre population depended only on the *change* of temperature and not on the temperature itself, i.e. the thermal sensitivity of the C_1 fibre population remained constant over a wide range of temperature. Hensel & Zotterman (1951*b*), on the other hand, working with single delta 'cold fibres', found distinct maxima in their individual thermal sensitivity curves such that in any particular fibre the response to a fixed cold stimulus was much greater at some temperatures than at others. There is no necessary difference in the behaviour of the C_1 and the delta fibres to cold, because the result we have obtained from the C_1 fibres reflects the behaviour of the *whole population*. In this population individual fibres may show sensitivity curves similar to those of the delta fibres, having distinct maxima, but provided these maxima are randomly distributed amongst the different fibres, over a limited range of temperature (as in our experiments) the integrated sensitivity curve of the whole population should be independent of temperature. On these same grounds the sensitivity should fall off at high and at low temperatures: these temperatures presumably lay outside the range (24–33° C) over which we specifically examined the sensitivity curve.

Although these non-myelinated fibres behave as 'cold fibres', they might equally well be described as 'touch fibres', for they are also sensitive to touch. While studies on individual fibres in this population might reveal different relative sensitivities to the two forms of energy, so that different fibres might be accordingly classified arbitrarily as 'cold fibres' or 'touch fibres', such a classification might tend to obscure the important point that most of these fibres can signal both cold *and* touch. It seems better to describe them as fibres which are both *cold-sensitive* and *mechano-sensitive*. This terminology has the merit that it not only describes more fully their receptor properties, but carries no implications about sensations which may arise from their activity.

The fact that the same C fibres respond to two distinct forms of quite innocuous stimulation is to be distinguished from the well-known phenomenon that nerve fibres may be excited by various forms of energy, provided it is sufficiently intense. This behaviour of a large group of C fibres is, of course, at variance with any rigid view of the specific irritability of sensory nerve endings. Such behaviour, however, is by no means unique. It has long been suspected from experiments on man that pressure receptors might be excited by cold. Thus, Hensel & Zotterman (1951*c*) point out that it has been known for many years that cold weights seem heavier

than warm ones (Weber's illusion), and that ether or chloroform applied to the skin not only feels cold, but gives the sensation of pressure. By recording action potentials from the cat's tongue, Hensel & Zotterman (1951*c*) showed that a small group of fairly large fibres, which they classified as mechanoreceptors, could be excited briefly by cooling the tongue some 10–15° C. In their experiments the 'cold fibres' (the small delta fibres) did not respond to mechanical stimulation and the large 'touch-and-pressure' fibres were not excited by cooling. In a recent study, however, Boman (1958) found that delta 'cold fibres' in the trigeminal nerve can be made to fire by pressing a hair firmly on the face, and still more recently Witt & Hensel (1959) have found fibres (whose size was not determined) in the cat's saphenous nerve which were also excited both by mechanical and by thermal stimulation. Our finding that many C fibres respond both to moderate cooling of the skin and to touching it is therefore not a singular one, but rather only a striking example of the ability of certain nerve fibres to respond to more than one form of energy at physiological intensities. The most remarkable example of this perhaps is the facial pit organ of the pit viper, which is an exceptionally sensitive thermoreceptor but also responds to mild mechanical stimulation (Bullock & Diecke, 1956).

The fact that we have found many C fibres sensitive to moderate mechanical stimulation and to cooling is perhaps less remarkable than the fact that we have not found C fibres that respond only to noxious stimuli and which might thus be considered as 'pain fibres', for pain is the modality which has been most closely associated with C fibres. On applying intense heat to the skin—one of the classical ways of eliciting pain—we have never observed any reduction of any of the C elevations which we can clearly distinguish, although Maruhashi *et al.* (1952) and Iggo (1958, 1959) have found some C fibres in cats' cutaneous nerves which were excited by heating the skin. If C fibres do mediate pain from the skin, as is generally believed, such 'pain fibres' can make up only a small proportion of the whole C fibre population. In our experiments, even under favourable conditions, we should not be able to detect reductions of the main C elevations of less than about 5 %—but even 5 % of the C population is still a large number of fibres. The fact that the sensation of pain is so poorly localized does indeed suggest that it may be mediated by a relatively small number of fibres.

It is now clear that when the skin is cooled or touched a large mass of fibres discharges in addition to the previously studied A fibres. The significance, for central nervous function, of the afferent discharges in the different groups of fibres remains to be established. While an obvious function is that of mediating the sensations of cold and of touch, some of

these fibres may be concerned not with sensation at all, but with thermoregulation or some other purely reflex activity.

The main conclusion to be drawn from the present experiments, and our previous experiments on the responsiveness of C fibres to touch, is that the C fibre population can no longer be regarded as devoted only to mediating painful stimuli, but rather must be considered as subserving a variety of afferent functions just as does the A fibre population. The fact that non-myelinated fibres far outnumber myelinated fibres in the cutaneous nerves of the cat and of man (Ranson, Droegemueller, Davenport & Fisher, 1934) strongly suggests that the role of C fibres in signalling innocuous stimuli is an important one.

SUMMARY

1. Experiments were done to see if the sensory non-myelinated fibres in cutaneous nerves discharge when skin temperature is changed.

2. A large proportion of the C fibres in the cat's saphenous nerve was found to be sensitive to moderate cooling of the skin: the most sensitive of the C fibres discharged when the skin temperature was lowered by about 3° C (from about 34° C) and most of them discharged when it was lowered by about 11° C.

3. The response of the cold-sensitive C fibres took a second or so longer to reach maximal intensity and diminished more slowly than the response of the delta cold fibres.

4. The maximal intensity of the C fibre discharge varied linearly with the logarithm of the *fall* in skin temperature.

5. No discharge in any group of C fibres was detected when the skin was gently warmed or was heated to burning. This shows that at most there can only be a relatively small number of non-myelinated 'warm fibres' and specific 'pain fibres'.

6. Most of the cold-sensitive C fibres were also mechano-sensitive and were excited by drawing a cotton swab across the skin.

7. A large mass of C fibres in a cutaneous nerve thus responds to innocuous thermal and mechanical stimulation of the skin. Since non-myelinated fibres far outnumber all myelinated fibres in cutaneous nerves, they clearly play an important part in signalling such innocuous stimulation.

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THE EFFECT OF OXYTOCIN AND VASOPRESSIN AND OF PHENYLALANYL^3-OXYTOCIN ON THE URINARY EXCRETION OF WATER AND ELECTROLYTES IN MAN

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At the present time it is generally held that the neurohypophysis secretes two hormones, oxytocin and vasopressin. The latter is important in most species and in both sexes in the maintenance of normal water balance by promoting water reabsorption from the distal renal tubule. The importance and actions of oxytocin are, however, less well understood. In the female it produces contraction of the pregnant uterus and milk ejection by the lactating breast, but in the male, where similar amounts of the hormone have been found (van Dyke, Adamsons & Engel, 1955), its function remains obscure.

Attempts have been made to demonstrate a physiological action of oxytocin on renal function, especially electrolyte excretion, in experimental animals. In the rat, Dicker & Heller (1946), Croxatto, Rosas & Barnafi (1956) and Brunner, Kuschinsky, Münchow & Peters (1957) produced evidence of increased excretion of sodium, chloride and, less certainly, potassium after injections of purified oxytocic extract of posterior pituitary gland. Brunner *et al.* (1957) found similar results with synthetic oxytocin. Brooks & Pickford (1958), who also found no difference between the actions of highly purified oxytocic extract and synthetic oxytocin, report that the effect of oxytocin on electrolyte excretion in the bitch varied with the rate of urine flow. At low rates of urine flow injections of oxytocin increased sodium, chloride and sometimes potassium excretion; but during water diuresis no such effect was seen. Because there is evidence (Harris, 1955) that under physiological conditions oxytocin and vasopressin are usually liberated simultaneously from the neurohypophysis, Brooks & Pickford (1958) tested mixtures of the two hormones in the bitch and then found that increased electrolyte excretion was seen at both high and low rates of urine flow. The effects of oxytocin on the rate of urine flow have been variable. In the rat increases of urine flow were observed by Croxatto *et al.* (1956) and Brunner *et al.* (1957) but Gyermek & Fekete (1955) describe an antidiuretic action of synthetic oxytocin.

Both van Dyke *et al.* (1955) and Brooks & Pickford (1958) found oxytocin to have an antidiuretic action in the dog.

There have been very few reports of the effect of oxytocin on renal function in man. Chalmers, Lewis & Pawan (1951) saw little antidiuretic effect and no change in sodium or chloride excretion after 50–200 m-u. Pitocin (pituitary extract; Parke, Davis) given intravenously to normal human subjects during water diuresis. Brunner *et al.* (1957) gave synthetic oxytocin subcutaneously in doses of 15–240 m-u./kg body weight to ten subjects, seven male and three female, during antidiuresis and failed to observe an increase in urine flow or the excretion of chloride or potassium.

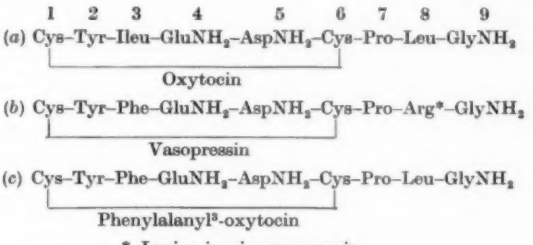

Fig. 1. Amino-acid structure of (*a*) oxytocin, (*b*) vasopressin and (*c*) phenylalanyl^3-oxytocin.

The structure of the neurophypophysial hormones, oxytocin and vasopressin, has recently been elucidated by du Vigneaud, Ressler, Swan, Roberts, Katsoyannis & Gordon (1953) and du Vigneaud, Gish & Katsoyannis (1954). Both hormones are cyclic octapeptides which differ from each other by only two amino acids (Figs. 1*a*, *b*). These differences in structure are associated with important differences in biological activity, one of which is the poor antidiuretic activity of oxytocin in animals (van Dyke *et al.* 1955) when compared with vasopressin. Thus a phenylalanyl group in the 3-position and/or an arginyl or lysyl group in the 8-position are necessary for the very potent antidiuretic effect of vasopressin. Analogues of these hormones have now been prepared in which individual amino acids are replaced by others. One such substance (Fig. 1*c*) called 'P-analogue' by Berde, Doepfner & Konzett (1957), 'oxypressin' by Katsoyannis (1957) and here called phenylalanyl^3-oxytocin according to the scheme of numbering the amino acids suggested by Konzett & Berde (1959), is of interest because it can be used to assess the relative importance of the amino acids in the 3- and 8-positions in the action of vasopressin on the kidney. It has been found by Berde *et al.* (1957) to have an antidiuretic activity in the rat comparable to that of vasopressin, whereas substitution of the tyrosyl^2 group of oxytocin by phenylalanine results in much

less enhancement of the antidiuretic action (Konzett & Berde, 1959). This suggests that the strong antidiuretic action is dependent on the exact structure of the cyclic pentapeptide part of vasopressin (Fig. 1).

In the experiments reported here the effects of oxytocin, vasopressin and mixtures of oxytocin and vasopressin on urinary excretion of water and electrolytes were studied in man. In addition, the actions of phenylalanyl[3]-oxytocin on renal function were compared with those of oxytocin and vasopressin.

METHODS

Nineteen healthy young males and one healthy young female were studied, the latter at a time one week after the cessation of a normal menstrual period. In each experiment an indwelling needle was inserted into a forearm vein and an infusion of sodium chloride solution (0·9 g/100 ml.) commenced, at a rate of 0·25 ml./min. After control urine collection for three periods of 10–15 min the infusion was changed to one containing the hormone and the chosen dose given usually during 10–20 min. Some of the males stood to pass urine but otherwise the subjects remained seated throughout the experiments. Urine was passed naturally every 10–20 min and the volume measured. Every effort was made to empty the bladder as completely as possible. Urine collections were continued for at least 60 min after the end of administration of each hormone. In some experiments, when urine flow had returned to control levels an infusion of another hormone was given and its effect followed as before. Water diuresis (when required) was induced by drinking 1 l. water 90 min before the start of the experiment. During the experiments water equal in amount to the urine passed was drunk after each collection period. Dilutions of the hormones with 0·9% saline were made less than 1 hr before their administration. Blood pressure was recorded by sphygmomanometer and pulse rate by counting every 5 min. Most experiments were performed in the morning but some took place in the afternoon. Urinary sodium and potassium concentrations were measured by flame photometry and in some experiments chloride concentration by the method of Sanderson (1952).

The hormone preparations which have been used in these experiments are: (*a*) 'Syntocinon' (Sandoz). This is a solution of synthetic oxytocin stated by the manufacturers to contain 10 international oxytocic units/ml.; in this paper the term 'milliunit' (m-u.) of oxytocin means 0·0001 ml. of 'Syntocinon'. (*b*) 'Pitressin' (Parke, Davis). This is a purified posterior pituitary extract which contains mainly vasopressin with a small amount of oxytocin which was considered immaterial to this study; it is stated to contain 20 international pressor units/ml. and in this paper the term 'milliunit of vasopressin' is used to mean 0·00005 ml. of 'Pitressin'. (*c*) A solution of phenylalanyl[3]-oxytocin, which was supplied by Sandoz Research Laboratories. The exact weight of analogue in solution is not known but when assayed by the makers the preparation had an antidiuretic activity in the rat of 6 u./ml. compared with U.S.P. Standard Posterior Pituitary Extract but only 1 u./ml. of pressor activity when assayed on the spinal cat. In this paper, dosage when expressed in m-u. refers to the antidiuretic activity in the rat of 6 u./ml.

The changes in electrolyte excretion occurring during and after these infusions have been expressed by taking the difference between the mean rate of excretion (μequiv/min) during the control period and the mean rate during antidiuresis, and expressing it as a percentage of the control rate. In assessing these results it should be remembered that small errors in emptying the bladder, especially when the rate of urine flow is low, produce substantial errors in the apparent rate of electrolyte excretion. For example, an error of only 2 ml. during a period of low urine flow can produce an error of 10–20% in apparent electrolyte excretion. It seems advisable therefore to regard changes of less than 40% as within experimental error.

RESULTS

Effects of vasopressin

Vasopressin was given during water diuresis to six male subjects, three receiving small doses (16, 22, 25 m-u.) and three relatively large doses (70, 100, 110 m-u.) (Table 1). All subjects had a definite antidiuresis lasting for more than 60 min. There was no significant change in sodium, potassium, or, in four subjects, chloride excretion. The apparently larger falls of sodium and potassium excretion in subjects R.F. and R.W. may be related to the marked reduction of urine flow, as suggested for a similar finding by Chalmers *et al.* (1951).

Effects of oxytocin

Oxytocin was given on eighteen occasions to sixteen male subjects and one female subject (N.M.) during water diuresis. A definite antidiuresis was seen in all except two experiments (Table 2). Subject A.B. who received 1100 m-u. over a period of 30 min had poor control of bladder emptying but the average rate of urine flow tended to decline throughout the experiment. Subject R.F. was studied on two occasions; when given only 100 m-u. of oxytocin no significant change in urine flow was seen, but in a later experiment, when he was given 1000 m-u. a definite antidiuresis was observed. When the hormone was given over a short period (10–20 min) the onset of antidiuresis occurred within 10 min of commencing the infusion and usually lasted only 20–40 min. From a comparison of Tables 1 and 2 it can be seen that the antidiuresis produced by doses of 200–3000 m-u. oxytocin is generally less than that produced by 16–110 m-u. vasopressin. When the antidiuretic actions of 500 m-u. oxytocin and 25 m-u. vasopressin were compared in the same experiment (Fig. 2) the effect of the vasopressin was much larger and more prolonged. In this experiment the hormones were given by intravenous injection lasting only 2 min so that the time of onset and the duration of the antidiuresis could be defined more clearly. From a comparison with Table 2 it can be seen that the antidiuretic effect of 25 m-u. vasopressin would be roughly matched by 2500 m-u. oxytocin (compare subject S.L. Table 1 and subject A.M. Table 2). The changes in electrolyte excretion were not significant (Table 2). In the fifteen subjects in whom it was measured chloride excretion closely followed that of sodium so that only the changes in sodium and potassium have been shown in the tabulated results.

Three male subjects were given oxytocin during periods of low urine flow (1–3 ml./min). There was no change in the rate of urine flow and no significant change in sodium or potassium excretion (Table 3).

TABLE 1. Effect of intravenous vasopressin on urine flow and on sodium, potassium and chloride excretion. (In this and subsequent tables urine flow 'during' hormone administration means the lowest rate of urine flow observed and electrolyte excretion 'during' means average rate of excretion during observable effect on rate of urine flow)

Subject (wt. kg.)	Dose (m-u.)	Urine flow (ml./min) Initial	During	After	Duration of anti-diuresis (min)	Electrolyte excretion (μequiv/min) Na Initial	During	Change (%)	K Initial	During	Change (%)	Cl Initial	During	Change (%)
V.L. (83)	16	14·6	4·0	13·6	75	259	245	− 5	63	60	− 5	179	196	+ 9
R.B. (61)	22	15·0	1·5	10·5	> 60	198	194	− 2	94	81	−14	269	253	− 6
S.L. (70)	25	14·2	1·8	10·5	> 60	207	223	+ 8	119	97	−18	223	230	+ 3
A.B. (66)	70	19·6	1·3	—	> 90	208	229	+10	79	93	+18	295	252	−15
R.F. (72)	100	15·8	1·0	—	> 90	523	292	−44	142	94	−34	—	—	—
R.W. (80	110	19·0	1·2	—	> 90	510	319	−37	189	115	−40	—	—	—

TABLE 2. Effect of intravenous oxytocin on urine flow and sodium and potassium excretion in 16 male and 1 female subjects during water diuresis

Subject (wt. kg.)	Dose (m-u.)	Urine flow (ml./min) Initial	During	After	Duration of anti-diuresis (min)	Electrolyte excretion (μequiv/min) Na Initial	During	Change (%)	K Initial	During	Change (%)
R.F.* (72)	100	20·6	20·2	19·2	—	573	598	+ 4	161	159	− 1
S.L. (70)	200	12·1	7·9	14·0	20	191	198	+ 4	204	182	−11
J.H. (66)	200	17·3	7·5	15·4	25	86	64	−26	71	63	−11
P.L. (74)	300	14·4	10·5	13·5	20	188	123	−35	126	95	−25
f N.M. (58)	500	11·1	4·5	9·5	35	197	187	− 5	69	55	−20
S.T. (89)	500	24·6	10·0	20·0	40	561	445	−21	245	242	− 1
W.T. (87)	500	11·3	3·4	12·0	30	154	157	+ 2	81	75	− 7
R.B. (69)	500	15·6	6·8	15·4	20	79	74	− 6	39	41	+ 5
G.F. (55)	500	12·6	6·1	13·9	30	323	304	− 6	87	111	+28
J.C. (66)	650	14·9	3·5	14·9	25	178	175	− 2	75	98	+30
D.H. (80)	750	7·6	1·7	10·3	25	214	205	− 4	106	123	+16
M.R. (89)	800	16·0	6·5	16·0	50	463	398	−14	124	130	+ 5
R.F.* (72)	1,000	18·8	5·4	15·3	60†	559	467	−16	162	163	+ 1
A.B. (66)	1,100	18·3	16·9	16·0	—†	151	107	−29	84	59	−30
D.C. (75)	1,250	16·5	1·7	13·2	25	210	159	−24	117	113	− 3
B.J. (72)	1,500	15·2	3·7	14·9	40	152	133	−12	144	104	−28
A.M. (55)	2,500	18·0	1·0	15·8	70†	259	235	− 9	161	125	−22
S.W. (54	3,000	12·5	1·4	10·8	45	107	115	+ 7	106	90	−15

* Subject studied on 2 occasions. † Infusion given over period of 30 min. *f*, female subject.

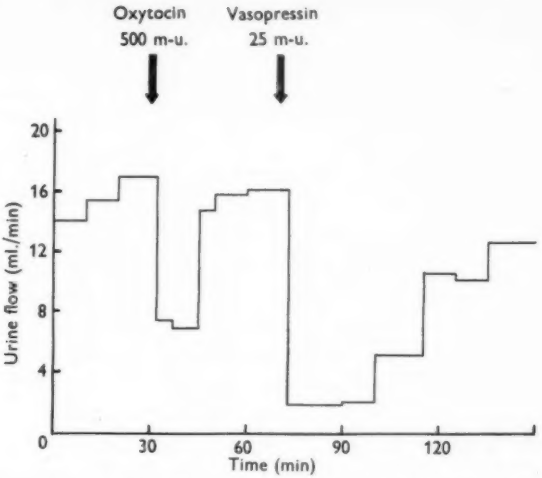

Fig. 2. Comparison of the effects of intravenous injection of oxytocin and of vasopressin on urine flow during water diuresis in the same experiment.

TABLE 3. Changes in electrolyte excretion in 3 subjects given oxytocin during a period of low rate of urine flow (1–3 ml./min)

		Electrolyte excretion (μequiv/min)					
		Na			K		
Subject (wt. kg.)	Dose (m-u.)	Before	During	Change (%)	Before	During	Change (%)
K.T. (71)	700	333	334	0	75	74	−1
W.T. (87)	750	226	230	+2	96	91	−3
J.R. (80)	800	177	187	+6	181	170	−6

Effects of mixtures of oxytocin and vasopressin

Mixtures of oxytocin and vasopressin, usually in the ratio of 25:1, were given to six male subjects and one female subject during water diuresis and to three male subjects during a period of low rate of urine flow. No significant changes in sodium or potassium excretion were seen (Table 4).

Effects of phenylalanyl[3]-oxytocin

Phenylalanyl[3]-oxytocin had a definite antidiuretic effect in all eight subjects to whom it was given (Table 5). The antidiuretic effect of oxytocin or vasopressin when tested as a continuation of the same experiment in these subjects is shown in Tables 1 and 2. The results in subjects R.B., S.L., and V.L. indicate that 0·0027–0·0042 ml. (16–25 m-u. of antidiuretic activity when compared with U.S.P. Posterior Pituitary Extract

TABLE 4. Changes in sodium and potassium excretion after mixtures of oxytocin and vasopressin during water diuresis (upper seven subjects) and at low urine flow (lower three subjects); *f*, female subject.

		Electrolyte excretion (μequiv/min)					
	Dose (m-u.)	Na			K		
Subject	Oxytocin:vasopressin	Before	During	Change (%)	Before	During	Change (%)
R.F.	50:50	451	318	−32	115	69	−40
G.F.	500:20	331	198	−40	112	84	−25
*f*N.M.	500:25	203	174	−14	52	228	−54
F.G.	520:25	199	195	− 2	118	115	− 3
D.H.	750:30	146	129	−12	158	130	−18
D.C.	1,500:60	201	219	+ 9	108	100	− 7
S.W.	2,500:50	139	136	− 2	56	40	−29
W.T.	750:30	165	108	−32	150	140	− 7
D.C.	800:40	246	281	+41	80	101	+26
S.W.	1,200:40	203	144	−29	141	117	−17

TABLE 5. Effect of intravenous infusions of phenylalanyl[3]-oxytocin on urine flow and electrolyte excretion in eight male subjects during water diuresis (subjects S.L. and V.L. studied on two occasions)

						Electrolyte excretion (μequiv/min)								
		Urine flow (ml./min)			Duration of anti-diuresis (min)	Na			K			Cl		
Subject (wt. kg.)	Dose (m-u.)	Initial	During	After		Initial	During	Change (%)	Initial	During	Change (%)	Initial	During	Change (%)
S.L. (70)	20	14·0	4·2	13·2	30	234	185	−21	225	210	− 7	157	111	−29
S.L.	25	9·0	1·1	14·2	40	162	169	+ 4	135	196	+45	108	122	+13
V.L. (83)	16	14·6	4·9	14·6	55	215	248	+13	153	192	+25	95	83	−13
V.L.	50	16·0	3·4	15·5	70	284	339	+19	—	—	—	80	93	+16
R.B. (61)	22	14·0	1·4	15·0	40	168	161	− 4	224	254	+13	74	88	+19
J.C. (66)	30	14·6	1·7	11·3	40	216	174	−19	231	222	− 4	123	113	− 8
S.T. (89)	40	22·6	8·3	24·3	25	727	718	− 1	—	—	—	180	243	+34
P.L. (74)	56	16·5	2·2	14·0	40	322	238	−28	—	—	—	114	127	+11
J.H. (66)	75	15·4	0·9	12·3	55	62	48	−23	108	88	−19	77	50	−35
W.T. (87)	25	12·0	2·2	9·5	>40	—	—	—	—	—	—	—	—	—

in the rat) of the solution of the analogue had an antidiuretic effect similar to 16–25 m-u. vasopressin, but the effect of vasopressin lasted rather longer. Doses of 200–650 m-u. oxytocin when compared with 0·0033–0·0125 ml. (20–75 m-u. of antidiuretic activity) phenylalanyl^3-oxytocin solution had a smaller effect (subjects W.T., S.L., J.C., J.H., P.H. and S.T.). The effect of 0·005 ml. (30 m-u. of antidiuretic activity) is more closely matched by 2500 m-u. oxytocin (Table 2) suggesting that in respect of their antidiuretic action in man 0·005 ml. of phenylalanyl^3-oxytocin solution, 2500 m-u. oxytocin, and 20 m-u. vasopressin are equally effective.

As with oxytocin and vasopressin the changes in electrolyte excretion produced by the analogue (Table 5) were insignificant and showed no consistent direction of change. No changes in heart rate or blood pressure were observed during any of these experiments.

DISCUSSION

Effects of vasopressin

The antidiuretic action of vasopressin is well known and is reported here mainly to provide a comparison for the antidiuresis observed with oxytocin and with phenylalanyl^3-oxytocin under similar experimental conditions. Although increases of sodium and potassium excretion after vasopressin have been reported in the rat (Anslow & Wesson, 1955) and the bitch (Brooks & Pickford, 1958) the results in Table 1 and those of Black & Thomson (1951), Chalmers *et al.* (1951) and Murphy & Stead (1951) indicate that it has no direct effect on electrolyte excretion in man.

Effects of oxytocin and of mixtures of oxytocin and vasopressin

The finding of a definite antidiuretic action of oxytocin in man does not appear to have been reported before. Chalmers *et al.* (1951) stated that they observed little antidiuretic effect after 50–200 m-u. Pitocin given intravenously to normal human subjects, although their figures do suggest a definite fall in urine flow in some subjects. In the present study the one subject who only received 100 m-u. oxytocin did not show any change in the rate of urine flow, but with doses of 200 m-u. or more only one out of seventeen failed to show a definite antidiuresis. It is difficult to relate the doses of oxytocin used in these experiments to the amounts secreted physiologically as neither the amount of the hormone released from the pituitary nor the normal blood levels are precisely known. One of the most accurate estimates of normal blood levels is that of Bissett & Lee (1957) who found oxytocic activity of up to 0·4 m-u./ml. in the peripheral blood of conscious subjects during cardiac catheterization. A rapid intravenous injection of 1000 m-u. oxytocin would give an approximate blood level of

0·2 m-u./ml. but with infusions lasting 10–20 min this level would not be attained owing to destruction or excretion of the hormone. Thus in these experiments the doses of oxytocin were such as might be released endogenously. However, oxytocin is so much less active than vasopressin as an antidiuretic that it would seem unlikely that it has any importance during physiological antidiuresis.

The mechanism of the reduction of urine flow by oxytocin is unknown. The similarity of the structure of the two hormones would suggest that oxytocin acts at the same site as vasopressin, namely the renal tubule. However, preliminary studies of endogenous creatinine clearances in three subjects and inulin and *p*-amino-hippuric acid clearances in one subject have indicated a reduction of glomerular filtration rate and renal blood flow, so that an action on the renal circulation may be at least a factor in reducing the rate of urine flow. This would be in contrast to the antidiuresis of physiological doses of vasopressin where no change of glomerular filtration rate or renal blood flow occurs (Maxwell & Breed, 1951).

One of the main reasons for undertaking this work was to see whether oxytocin and, in view of the work of Brooks & Pickford (1958) in the bitch, more especially mixtures of oxytocin and vasopressin have any action on urinary electrolyte excretion in man. Like Chalmers *et al.* (1951), who used rather smaller doses, and Brunner *et al.* (1957), who used larger doses but by the subcutaneous route, I have been unable to demonstrate any increase in sodium, chloride or potassium excretion after the administration of oxytocin. This was so whether the rate of urine flow was high or low. Similarly, mixtures of oxytocin and vasopressin also failed to reveal any consistent change in urinary electrolyte excretion either at high or low rates of urine flow, although doses of the hormones used in this study are of the same order, on a body weight basis, as those used by Brooks & Pickford (1958). The difference in the results cannot be attributed to a difference in sex of the experimental subjects, since the one female in this series and the three studied by Brunner *et al.* (1957) gave results no different from the males. Brooks & Pickford (1958) give evidence that the effect of oxytocin which they observed was not due to a direct action on the kidney, but involved some central response to the hormone, and this may be the explanation of a difference between the two species.

Effects of phenylalanyl³-oxytocin

Since the recognition of the structure of the neurohypophysial hormones it has been important to relate their structure to their biological actions. These results offer some confirmatory evidence in man of the suggestion by Berde *et al.* (1957) and Konzett & Berde (1959) from animal experi-

ments that the intact pentapeptide ring of vasopressin is essential to its strong antidiuretic activity. The relatively poor antidiuretic action of oxytocin appears to be related to the alteration of the structure of this ring.

SUMMARY

1. The effect of intravenous infusions of the neurohypophysial hormones oxytocin and vasopressin and of the phenylalanyl[3] analogue of oxytocin on urinary excretion of water, sodium, chloride, and potassium has been studied in seventeen male and one female normal human subjects.

2. Oxytocin in doses of 200–3000 m-u. was found to have a significant antidiuretic effect on a water diuresis.

3. No consistent changes in urinary electrolyte excretion could be demonstrated with either vasopressin, oxytocin or mixtures of the two. This was so at both high and low rates of urine flow.

4. Phenylalanyl[3]-oxytocin has been found to have the high antidiuretic activity in man which would be expected from its known activity in the rat. It had no significant effect on urinary electrolyte excretion.

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THE RELATION OF CIRCULATING NORADRENALINE TO THE EFFECT OF SYMPATHETIC STIMULATION

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From a comparison of the action of sympathomimetic amines in normal animals and in animals treated with reserpine, we were led to the conclusion that some of the simpler amines, such as tyramine, act by liberating noradrenaline from a store in the neighbourhood of sympathetic nerve endings (Burn & Rand, 1958*b*). The presence of noradrenaline in the walls of arteries and veins was demonstrated by Schmiterlöw (1948) and by von Euler & Purkhold (1951) in the spleen, the liver, the kidneys and the salivary glands. Treatment of animals with reserpine has been shown to cause a disappearance of the extractable noradrenaline from the heart (Bertler, Carlsson & Rosengren, 1956) and from the blood vessels, spleen and iris (Burn & Rand, 1957, 1958*a*, 1959). Sympathomimetic amines such as tyramine lost their action in animals treated with reserpine, but this action was restored following an infusion of noradrenaline into a vein. We concluded that the infusion of noradrenaline had replaced some of the noradrenaline in these organs.

In order to obtain information on the role of the noradrenaline normally present in the vessel walls, we have studied the vasoconstriction caused by sympathetic stimulation before and after the infusion of noradrenaline.

METHODS

Experiments have been made on dogs anaesthetized with chloralose 80 mg/kg after preliminary ether anaesthesia. After evisceration the lumbar vessels and sympathetic chain were divided between ligatures at the level of L4. The peripheral end of the sympathetic chain was threaded into the electrodes shown in Fig. 1, which were designed and made by Mr O. B. Saxby. The part of the chain enclosed in the electrodes was irrigated throughout the experiment with oxygenated Krebs's solution flowing from a reservoir at 37° C through the polythene tube E at a rate controlled by a drip. As the irrigating solution left the electrodes it was removed by another tube connected to a suction pump. For stimulation, square wave pulses of 2 msec pulse width were applied at a rate of 25/sec for periods of 10 sec or less. Vasoconstriction was recorded by measuring either volume changes or the venous outflow during perfusion of the leg.

Plethysmographic recording. The right hind leg of the dog was enclosed in a metal plethysmograph with a thin latex lining, filled with water at 37° C and connected to a piston recorder sufficiently sensitive to record the change in volume of the leg with each heart beat.

In some experiments blood was collected from a second dog into a vessel containing heparin, and put into a Marriotte bottle of 1 l. capacity. A cannula of as wide diameter as possible was tied in the left external iliac artery of the dog used for the experiment and was connected to the bottom opening of the Marriotte bottle. The air space above the blood in the bottle was continuous with the air in a 20 l. stone bottle, in which an air pressure was maintained equal to the dog's blood pressure. This device prevented variations in the dog's blood pressure.

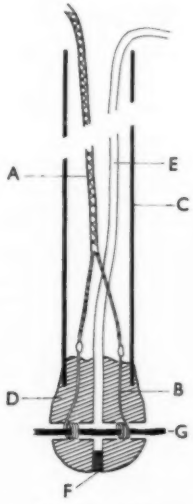

Fig. 1. Diagram of electrodes. A is twin 7/42 copper, flat laid, screened PVC sheathed cable. B is platinum wire, 0·35 mm. C, is a glass tube 20 cm long, outside diameter 6 mm. E is polythene tube 1 mm bore (Allen and Hanburys, No. 2). F is a cleaning hole, normally plugged. G is the nerve.

Perfusion of hind leg and recording of venous outflow. A dog under ether anaesthesia was bled into a vessel containing heparin and its lungs were then perfused with the blood, with a Dale–Schuster pump. This perfusion was continuous until the leg of a second dog was ready. The second dog was eviscerated under ether, and a cannula was tied in the left external iliac artery pointing to the bifurcation of the aorta. The aorta itself was tied below the origin of the two external iliac arteries and ligatures were also put in place around the aorta above the bifurcation. The right sympathetic chain was prepared for stimulation as already described, and mass ligatures were put in position so as to enclose the whole cross-section of the body wall about the level of the kidney. At a given moment the aorta was tied above the bifurcation, and a cannula was then tied in the inferior vena cava. The perfusion of the right hind leg was begun through the left external iliac artery by means of a second Dale–Schuster pump. The delay between arrest of the natural circulation and the start of the perfusion was usually 3 min. The mass ligatures were tied. The venous outflow was recorded with a Stephenson (1949) recorder. When the sympathetic chain was stimulated, constriction was recorded by the rise of pressure in the arterial cannula and by the fall in venous outflow. No anaesthetic was present during the observations; they were made 1–2 hr after the ether used during the preparation had been discontinued.

RESULTS

Experiments with atropine

Plethysmographic recording. Since the effects of sympathetic stimulation in the dog's hind leg include vasodilator as well as vasoconstrictor changes (Burn, 1932*a*), the experiments were first made in the presence of atropine so as to exclude the vasodilatation produced by the cholinergic sympathetic fibres. The results shown in Fig. 2 illustrate the findings. At (*a*) the effect of stimulating the lumbar sympathetic chain for 10 sec is shown. When the strength of current was 0·3 mA, there was no effect;

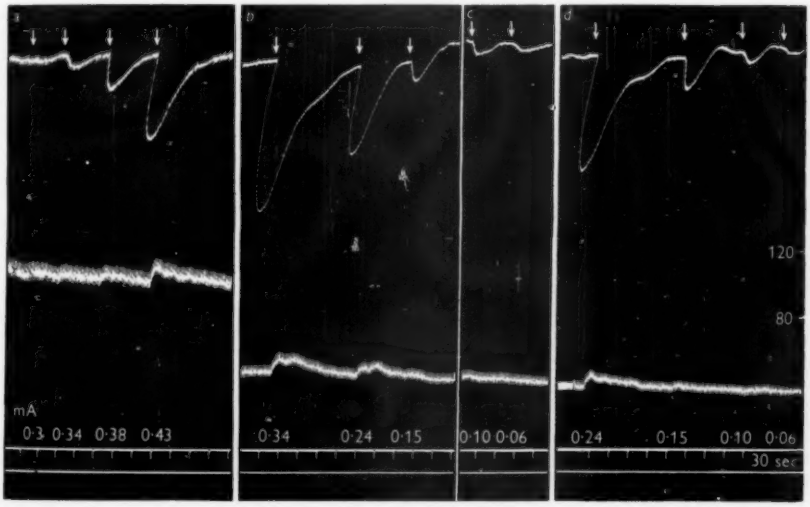

Fig. 2. Dog under chloralose. The upper record shows changes in volume of the right hind leg. At the arrows the right lumbar sympathetic chain was stimulated for 10 sec with square-wave stimuli of frequency 25/sec, duration 2 msec and of strength shown at the foot of the record. The lower record is the blood pressure. Observations were made in the presence of atropine. (*a*) shows that initially the threshold strength was 0·34 mA. Between (*a*) and (*b*) an infusion of 0·2 mg noradrenaline was given. (*b*) shows that the effect of 0·34 mA was greatly increased and (*c*) shows that the threshold was 0·06 mA. (*d*) shows that 30 min later the effects of 0·24 mA and of 0·15 mA were greater than in (*b*).

at 0·34 mA there was a trace of constriction; at 0·38 and 0·43 mA the constriction was progressively greater. The threshold strength therefore was 0·34 mA. Between (*a*) and (*b*) an intravenous infusion of 0·2 mg noradrenaline was given during 20 min. Sympathetic stimulation was then much more effective. The previous threshold strength, 0·34 mA, now

caused a large vasoconstriction, and the threshold strength (*c*) was reduced to 0·06 mA. After a further period of 30 min the effect of 0·24 mA, shown in (*d*), was greater than in (*b*), but the threshold remained at 0·06 mA.

In the experiment described there was a rise of blood pressure during the infusion of noradrenaline; when the infusion was stopped the blood pressure fell to a lower level than before the infusion. However, the same result was obtained when these pressure changes were prevented by the use of a constant-pressure reservoir connected to the left external

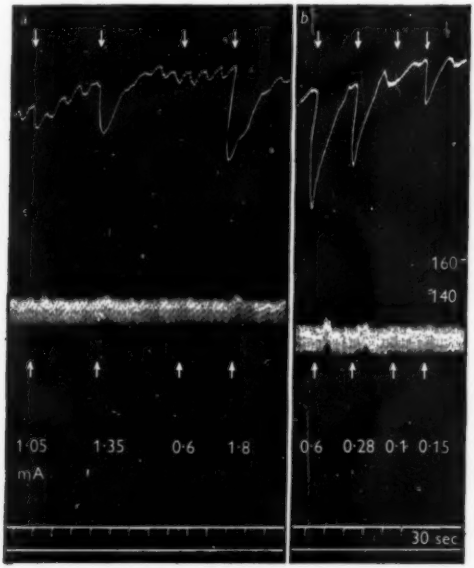

Fig. 3. Observations as in Fig. 2. In this experiment the blood pressure was kept approximately constant during and after the infusion of 1·0 mg noradrenaline by connecting the left external iliac artery to a reservoir of blood under pressure. (*a*) threshold strength was 1·05 mA before the infusion of noradrenaline. (*b*) threshold strength was 0·15 mA after the infusion.

iliac artery. Such an experiment is shown in Fig. 3. Before the infusion of noradrenaline (at *a*) the threshold strength was 1·05 mA and 0·6 mA was without effect. Greater effects were observed with 1·35 and 1·8 mA. After the infusion of 1·0 mg noradrenaline (at *b*) the threshold was reduced to 0·15 mA and the effect of 0·6 mA was greater than that of 1·8 mA before the infusion.

Table 1 shows the reduction of the threshold observed in seven experiments in which the plethysmograph was used, the threshold being reduced

to a percentage of the initial value varying from 14 to 75. In many experiments it was observed that the greatest fall in threshold did not occur immediately the infusion of noradrenaline was terminated, but after a delay of 30–60 min.

Perfusion of hind leg and recording of venous outflow. The effect of noradrenaline infusion is shown in Table 1 and Fig. 4. Table 1 shows the fall in thresholds in two experiments after noradrenaline infusion. Figure 4 shows an experiment in which the same strength of stimulation, 3·2 mA, was applied throughout. The effect of stimulation was small and declined during the course of the perfusion. The effects of the last two stimulations before the noradrenaline infusion are shown at (*a*) and (*b*). Between (*b*) and (*c*) 0·15 mg noradrenaline was infused. When the infusion was stopped

TABLE 1. Effect of noradrenaline infusion on threshold strength of sympathetic stimulation for vasoconstriction. Observations in presence of atropine

Type of experiment	Initial threshold strength (*A*) (mA)	Noradrenaline infusion (mg)	Threshold strength after infusion (*B*) (mA)	Threshold *B* as % of threshold *A*
Plethysmograph	1·05	1·0	0·15	14
	0·77	1·0	0·38	50
	1·16	0·025	0·87	75
	1·02	0·2	0·27	26
	0·42	0·175	0·27	64
	0·19	0·25	0·08	42
	0·34	0·2	0·06	29
Perfusion	1·28	3·2	0·92	72
	0·79	0·64	0·51	64

and the pressure in the arterial cannula had once more returned to its initial level, stimulation had an increased effect, but with repetition of stimulation the effect became progressively weaker (*c*). This decline of the effect was not observed in the plethysmograph experiments on dogs under chloralose and may be explained if the uptake of noradrenaline was relatively small for lack of some other substance in the perfusion circuit necessary to hold noradrenaline in the store. Thus, in the adrenal medulla noradrenaline and adrenaline are held in granules in which they are bound to adenosine triphosphate (Blaschko, Born, D'Iorio & Eade, 1956).

Effect of adrenaline. In three plethysmograph experiments 0·25 mg adrenaline was infused instead of noradrenaline. There was a fall of threshold to 80% in one experiment, no change in a second, and a rise in a third experiment in which a fall of threshold was subsequently produced by an infusion of noradrenaline. Thus adrenaline was less effective than noradrenaline in lowering the threshold. A similar difference was obtained in experiments in which restoration of the pressor action of tyramine was investigated (to be published).

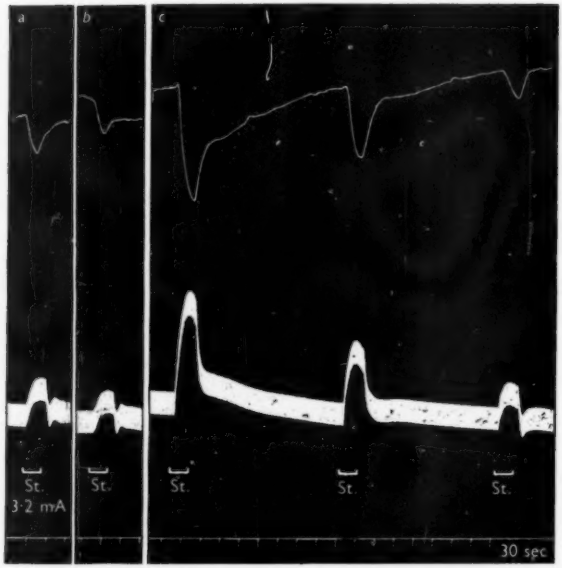

Fig. 4. Perfusion of dog's hind leg. (*a*) and (*b*) show effect of stimulating lumbar sympathetic chain for 0·5 min with a frequency of 25/sec, duration 2 msec and strength 3·2 mA. Between (*b*) and (*c*) an infusion of 0·15 mg noradrenaline was given. (*c*) shows the greater effect of the same stimulation, the increase diminishing with successive stimulations.

Experiments without atropine

Figure 5 illustrates a plethysmograph experiment in which the effect of an infusion of noradrenaline was examined without the use of atropine. The effect of stimuli of increasing strength before the noradrenaline infusion are shown at (*a*) and (*b*). A stimulus of 0·6 mA caused vasodilatation; one of 1·0 mA caused dilatation interrupted by constriction; and stimuli of 1·8 and 2·25 mA caused constriction only. After the infusion of 1 mg noradrenaline, during which the blood pressure was maintained constant by the use of the reservoir, the stimuli of 0·6 and 1·0 mA both caused dilatation followed by constriction. The stronger stimuli of 1·8 and 2·25 mA both caused constriction followed by dilatation, the constrictor phase being less than the constriction caused by the same stimuli before the noradrenaline infusion. Thus the infusion of noradrenaline appeared to increase the dilator effect of these stimuli to an extent which masked the increase in the constrictor effect.

In perfusion experiments in which atropine was not given the results of

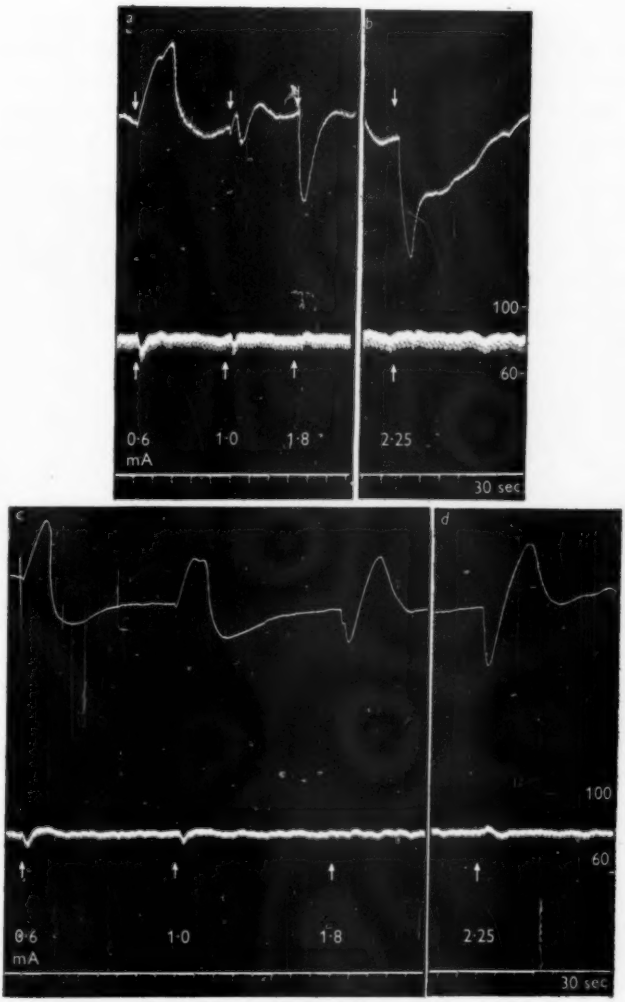

Fig. 5. Observations similar to those in Fig. 3 but made without atropine. (*a*) and (*b*) show the change in the response with increasing strength of stimulation from dilatation to constriction. Noradrenaline 1·0 mg was then infused. In (*c*) the effects of 0·6 mA and 1·0 mA were now dilatation followed y constriction, the effects of 1·8 and 2·25 mA were constriction followed by dilatation.

sympathetic stimulation were often a mixture of constriction and dilatation as is shown in Fig. 6. In (*a*) stimulation caused initial constriction, then dilatation and then further constriction. After the infusion of noradrenaline (*b*) the stimulation caused a more prolonged constriction without dilatation, which was probably masked. This result is consistent with the view that the noradrenaline infusion increases the constrictor response.

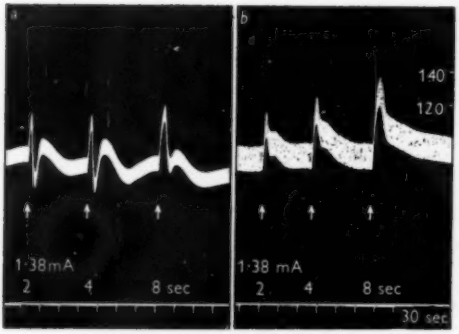

Fig. 6

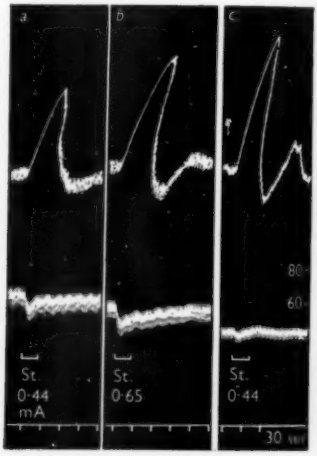

Fig. 7

Fig. 6. Perfusion of dog's hind leg. (*a*) initial responses were constriction with an intermediate phase of dilatation. (*b*) after the infusion of noradrenaline 0·25 mg. the constriction was now greater and masked the phase of dilatation.

Fig. 7. Plethysmograph observations on the dog's hind leg after removing the skin. (*a*) strength of stimulus 0·44 mA, causing dilatation only. (*b*) 0·65 mA dilatation followed by constriction. (*c*) 0·44 mA applied after a 0·25 mg noradrenaline infusion caused a greater dilatation than in (*a*), followed by constriction.

Since Bülbring & Burn (1935) showed that the dilator response to sympathetic stimulation was greatest in the skinned limb, we have also made observations in the hind leg of the dog after removing the skin. Figure 7 shows the plethysmograph record of a skinned leg, which responded to the stimulus of 0·44 mA strength with dilatation (*a*), and to the stimulus of 0·65 mA with a slightly greater dilatation followed by constriction (*b*). After an infusion of 0·25 mg noradrenaline, stimulation with 0·44 mA caused a still greater dilatation followed by constriction (*c*). Thus the noradrenaline infusion again increased both the dilator and the constrictor phase of the response.

DISCUSSION

The experiments described in this paper were prompted by observations (Burn, 1932*a*) that the vasoconstrictor effect of sympathetic stimulation in the perfused hind leg of the dog was increased when adrenaline was added to the perfusing blood. The effects observed were not great and were in the main found only when the adrenaline was actually present (Burn, 1932*a*). In more recent work we have found that when noradrenaline was used instead of adrenaline the constrictor action of tyramine was increased after the addition of noradrenaline had stopped and when its direct effect on the vessels had disappeared (Burn & Rand, 1958*b*). The present experiments have shown that, when vasodilator effects were excluded by atropine, the threshold strength of stimulus applied to the sympathetic chain for vasoconstrictor effects was reduced in some experiments to as little as one-seventh of the previous value by an infusion of noradrenaline.

This change in threshold is an unusual phenomenon, which cannot be explained by any change at the site of application of the stimulus, since there was no circulation of blood through the chain at that point. The number of nerve fibres excited must therefore have been the same although the threshold fell after noradrenaline was infused. The following suggestion put forward earlier by one of us (Burn, 1932*b*) for adrenaline is likely to apply to the results now obtained if noradrenaline is substituted for adrenaline: 'If it is true that, when the sympathetic nerves are stimulated, adrenaline is liberated from the endings, then it is necessary to suppose that there is a store of adrenaline in the neighbourhood of the endings ready to be liberated when the sympathetic impulse arrives.... If now adrenaline is added to the circulation it may be supposed that the magazine or store at the end of each sympathetic nerve is replenished....'

In 1948 Schmiterlöw showed that noradrenaline could be extracted from blood vessels and in 1951 von Euler & Purkhold extracted it from the spleen, the kidney and other organs. This extractable noradrenaline is presumably the store. Von Euler (1956) has expressed the view that it is contained within the nerve terminations, since 'no evidence has been obtained for the assumption that noradrenaline may be located outside the nervous tissue itself.' This conclusion however led him to suppose that these nerve terminals might contain as much as 3–30 mg/g although the fibres of the splenic nerve outside the spleen contain only 15 μg/g. The view that the store of noradrenaline is within the nerves affords the simplest explanation for the disappearance of the store when the nerves degenerate (von Euler & Purkhold, 1951).

Another possibility has arisen from the finding of chromaffin cells in

human skin (Adams-Ray & Nordenstam, 1956; Burch & Phillips, 1958). Mr E. H. Leach has found similar cells in the skin of the rabbit ear (see Burn & Rand, 1958*a*), in the cat's nictitating membrane and in the arrectores pili muscles of the cat's tail. These cells disappear or lose their granules after the administration of reserpine or when the sympathetic nerves have degenerated, and in both circumstances the store of extractable noradrenaline disappears (Burn, Leach, Rand & Thompson, 1959). It is thus a possibility that the store of noradrenaline in the neighbourhood of sympathetic nerve endings is present in chromaffin cells.

Whatever be the precise nature and location of the store, the fact that it can be replenished from noradrenaline circulating in the blood means that the post-ganglionic sympathetic adrenergic nerve endings differ from the cholinergic nerve endings at the neuromuscular junction, where the acetylcholine seems to be produced by the nerve terminal exclusively. Thus at the post-ganglionic sympathetic nerve ending there is a mechanism for taking up circulating noradrenaline as well as for releasing it.

We see in these observations evidence of a possible function for the noradrenaline secreted into the blood by the adrenal gland. There has hitherto been no satisfactory explanation of this secretion and it now appears that it might be secreted in order to fill up the stores at the sympathetic nerve endings. Adrenaline is much less effective as regards this function. We can see further that, if the activity of the adrenal medulla in releasing noradrenaline is excessive, the tone maintained by sympathetic impulses may also become excessive.

The evidence further suggests that when noradrenaline disappears from the blood the disappearance may be partly due to its uptake and storage. Until now the natural assumption has been that when noradrenaline disappeared it was destroyed. This, however, is no longer the only possibility.

SUMMARY

1. Experiments have been carried out in which the threshold strength of stimulation of the lumbar sympathetic chain for producing vasoconstriction in the vessels of the dog's hind leg was determined. Vasoconstriction was measured by using a plethysmograph in the eviscerated and anaesthetized dog, and also by changes in arterial resistance and venous outflow in the perfused preparation.

2. After an infusion of noradrenaline had been given and after the direct effect of the infusion had disappeared, it was found that the threshold of sympathetic stimulation was reduced, often considerably, and that the effect of a given strength of stimulus was increased. An infusion of adrenaline had little or no effect.

3. It is suggested that the extractable noradrenaline present around sympathetic nerve endings forms a store from which noradrenaline is released by a sympathetic impulse. This store can also take up noradrenaline from the blood. The fall in the threshold following an infusion of noradrenaline suggests that before the infusion noradrenaline released by stimulation is at once returned to the store and is thus not available for an action on the effector organ, whereas after an infusion, when the store is full, the noradrenaline released is not taken up and therefore exerts an action.

This work was done during the tenure by one of us (M.J.R.) of a Fellowship from the Life Insurance Medical Research Fund of Australia and New Zealand. We are greatly indebted to Mr H. W. Ling for his very valuable assistance.

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THE EFFECTS OF γ-AMINOBUTYRIC ACID AND OTHER COMPOUNDS ON STRUCTURES OF THE MAMMALIAN NERVOUS SYSTEM WHICH ARE INHIBITED BY FACTOR I

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The inhibitory effects of extracts of brain tissue on certain mammalian synapses, both peripheral and central, have been described by Florey & McLennan (1955 *a*, *b*). They showed that extracts containing Factor I, which was first recognized by Florey (1954) for its ability to inhibit the discharge of the crayfish stretch receptor neurone, could cause a reversible block of transmission in sympathetic ganglia and in the monosynaptic reflex pathway of the spinal cord. Whereas the extract had to be applied topically to the exposed cord, ganglionic transmission could be blocked *in vivo* by close arterial injection into the ganglia or *in vitro* by topical application.

Since then attempts have been made to identify the chemical entity responsible for these and other actions displayed by Factor I. Bazemore, Elliott & Florey (1957) adduced evidence for the presence in their extracts of γ-aminobutyric acid (GABA), and showed further that this substance was highly active in blocking the crayfish stretch receptor neurone discharge. McLennan (1958) has demonstrated, however, that GABA is not always present in brain extracts possessing Factor I activity and he has also shown (1959) that paper chromatographic separation of Factor I yields two active fractions. The chromatographic characteristics of one of these fractions are similar to those not only of GABA but also of β-guanidinopropionic acid (BGPA) and of γ-guanidino-butyric acid (GGBA), and the suggestion was made that these compounds might be present in the brain extracts as active ingredients when GABA appeared to be absent. It was established that both these guanidino acids are able to inhibit the crayfish stretch receptor neurone in concentrations approaching those required with GABA (see also, Kuffler & Edwards, 1958). Purpura, Girado, Smith, Callan & Grundfest (1959) have also demonstrated that the effects of these acids on the induced responses of the cerebral cortex are similar to those produced by GABA.

Two other related compounds with certain inhibitory properties have been described in the literature. The first is L-γ-amino-β-hydroxybutyric acid, which according to Hayashi (1959) is some fifty times more potent than GABA in preventing seizures induced by several different means. However, McLennan (1957) compared GABA and DL-γ-amino-β-hydroxybutyric acid in a number of situations and concluded that the latter acted qualitatively very similarly to GABA and was only about half as active. The other substance which has recently been shown to have some inhibitory actions is γ-amino-butyrylcholine (GABCh). It has been reported by Kuriaki, Yakushiji, Noro, Shimizu & Saji (1958) to be present in brain. Takahashi, Nagashima & Koshino (1958) have reported that its topical application to the brain results in depression of evoked potentials in the sensory cortex and of the 'dendritic' potentials observed with direct cortical stimulation.

In view of the fact that GABA, BGPA and GGBA may all be constituents of Factor I preparations, we have compared their actions, and those of GABCh, with those of Factor I on different structures of the mammalian nervous system. The results of this work indicate that none of these compound can account for all the actions obtainable with Factor I preparations.

METHODS

Purification of Factor I

One kilogram of fresh ox brain was boiled in water and dialysed, and the dialysate was concentrated *in vacuo* and treated with charcoal and perchloric acid (Florey & McLennan, 1955*b*). The concentrated dialysate was brought to pH 2·5 with HCl and poured into 10 vol. of absolute ethanol. The resulting precipitate was removed by centrifugation and the alcoholic solution was reduced to dryness *in vacuo*.

Separation of the two active fractions of Factor I (McLennan, 1959) was achieved by applying an aqueous solution of the alcoholic residue obtained above to a cellulose column (60 cm by 2·5 cm diameter) and developing it with the top layer of an *n*-butanol:acetic acid:water (4:1:5 by vol.) solvent mixture. Effluent fractions of 10 ml. were collected. Portions (0·1 ml.) of these fractions were evaporated to dryness, dissolved in crayfish solution (van Harreveld, 1936) and assayed on the crayfish stretch receptor neurone (Florey, 1954). Concentrations of Factor I solutions are expressed in this paper in 'crayfish units reference' (c.u.r.) as defined by Elliott & Florey (1956). A reference standard Factor I solution was prepared by boiling 100 g of ox brain in 100 ml. of water, homogenizing, and centrifuging the homogenate. The supernatant fluid was kept frozen except when samples of it were required for assay; for most crayfish this standard could be diluted one hundredfold and still produce inhibition of the stretch receptor neurone. It was, therefore, arbitrarily assigned an activity of 100 c.u.r./ml. and all Factor I activities expressed in this paper are referred to this standard preparation.

The effluent fractions from the cellulose column possessing Factor I activity fell into two distinct groups. The fractions in each group were pooled and portions were developed on two-dimensional paper chromatograms. The solvents used were *n*-butanol:acetic acid:water (4:1:5 by vol.) mixture and 80% aqueous phenol, and the positions taken up by the Factor I activity were measured by cutting up and eluting the developed

chromatograms (McLennan, 1959), Fraction A had R_f values in these two solvent mixtures of approximately 0·2 and 0·3, respectively, and fraction B 0·45 and 0·8. The pooled fractions from the columns were taken to dryness and dissolved in Locke's solution for application to the mammalian nervous system preparations.

Nerve preparations

Monosynaptic reflexes were elicited in the cat anaesthetized with chloralose by applying electrical stimuli (3 V, 0·1 msec, 1/10 sec) to a single dorsal rootlet. The femur was fixed with a pin and the movements of the lower leg were recorded with a spring myograph on a smoked drum. The vertebral column was opened from the dorsal side between L 1 and L 7 and the spinal cord was exposed by incising the dura. The cord was gently hooked up on thin glass rods to ensure that applied solutions reached all parts of it.

The dissection of the inferior mesenteric ganglion of the rabbit and the method for recording from it *in vitro* have been described by Brown & Pascoe (1952). Stimulating electrodes were placed either on the inferior splanchnic or on the ascending mesenteric nerve, and recording electrodes on the ascending mesenteric nerve. One drop of the solution to be tested was applied to the ganglion. Maximal square-wave stimuli (15–20 V, 0·5 msec) were applied to the preganglionic nerve at a frequency of 30/min.

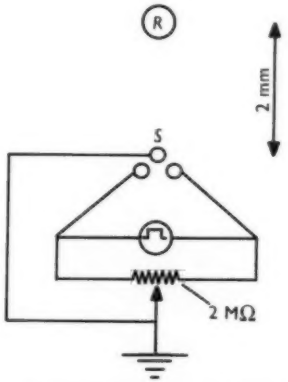

Fig. 1. The arrangement of electrodes used for stimulating the cortex and recording the evoked response a short distance away. The three stimulating electrodes (*S*) were of coated nichrome wire, 100 μ diameter; the single recording lead (*R*) was fixed in position 2 mm from the stimulating electrodes, and was of silver, 150 μ diameter.

Evoked potentials from the sensory cortex and the post-synaptic response of the nucleus gracilis were recorded from cats anaesthetized with Dial (allobarbitone; Ciba). The medulla oblongata and rostral end of the spinal cord were exposed and the cord was laid bare in the region of the second cervical root. Stimulating electrodes were applied to the dorsum of the cord at the lower level, and a monopolar recording electrode to the exposed nucleus gracilis. The skull overlying the contralateral sensory area of the cortex was removed and a second monopolar recording electrode was placed on the exposed brain. Maximal stimuli (3–4 V, 0·1 msec) were applied to the dorsal columns at a frequency of 1/sec and solutions were tested by placing two drops close to the recording electrode on the nucleus.

Direct cortical responses were recorded from the motor area in anaesthetized cats. The electrode assembly shown in Fig. 1 was placed on the exposed cortex and two drops of the solution to be tested were applied in the vicinity of the recording lead.

All electrical responses were displayed on a double-beam oscilloscope from which records were obtained photographically. A direct-writing oscillograph with a frequency response flat to 3000/s and a paper speed of 100 mm/sec was also used.

RESULTS

Directly evoked cortical potentials

Iwama & Jasper (1957) and Purpura *et al.* (1959), who studied the effects of topically applied solutions of GABA on the response evoked in the cerebral cortex by stimulation of a nearby point, found that application of a 1% GABA solution resulted in abolition of the surface negative response, leaving only a positive deflexion. Purpura *et al.* have further reported that this property is possessed also by β-alanine, guanidinoacetic acid and BGPA, and that GGBA reduced the negative phase of the response and eliminated the appearance of the positive wave as well.

These results have been essentially confirmed in the present study except that little inhibitory effect of GGBA on the surface positive wave was noted. Because of the difficulty of interpreting the positive potential changes (see Elliott & Jasper, 1959) only changes in amplitude of the surface negative wave were measured, as this has been shown to be due to excitation of structures near the cortical surface (Adrian, 1936).

The results which we have obtained with inhibitory substances which block the surface negative waves are illustrated in Fig. 2. Within 10 sec of applying the solutions to the region of the recording electrode there was a drop in amplitude of the negative wave, due in part to the short-circuiting effect of the Locke's solution. This effect was of brief duration only. Thereafter, when the applied solutions contained inhibitory substances, the amplitude of the potential continued to decline, followed, in those cases where inhibition was not great, by partial recovery after some 3–4 min. In all cases rinsing the cortical surface with Locke's solution led to full restoration of the initial amplitude within 5–30 min. Application to the cortex of a 1% solution of GABA (equivalent to 3000 c.u.r. of Factor I/ml.) caused an immediate and sustained depression of the potential (◑ in Fig. 2), and the same effect was observed after the application of 1% solutions of GGBA or of BGPA (each the equivalent of 1000 c.u.r./ml.) Contrary to the report of Takahashi *et al.* (1958) no effect could be observed with GABCh, even in a 1% solution. Both fractions of Factor I in concentrations of about 700 c.u.r./ml. caused sustained reductions in the amplitude of the negative wave. Fraction A apparently had a more profound action on the cortex, since a threefold dilution resulted in only a small diminution of its inhibitory action, whereas the same dilution of fraction B reduced its activity to such an extent that the effect was

not only smaller, but also no longer sustained. Dilution of GABA to 300 c.u.r./ml. reduced its effectiveness in a similar manner.

Figure 2 illustrates the results of a typical experiment in which each inhibitory solution was applied to the cortex three times in a random order

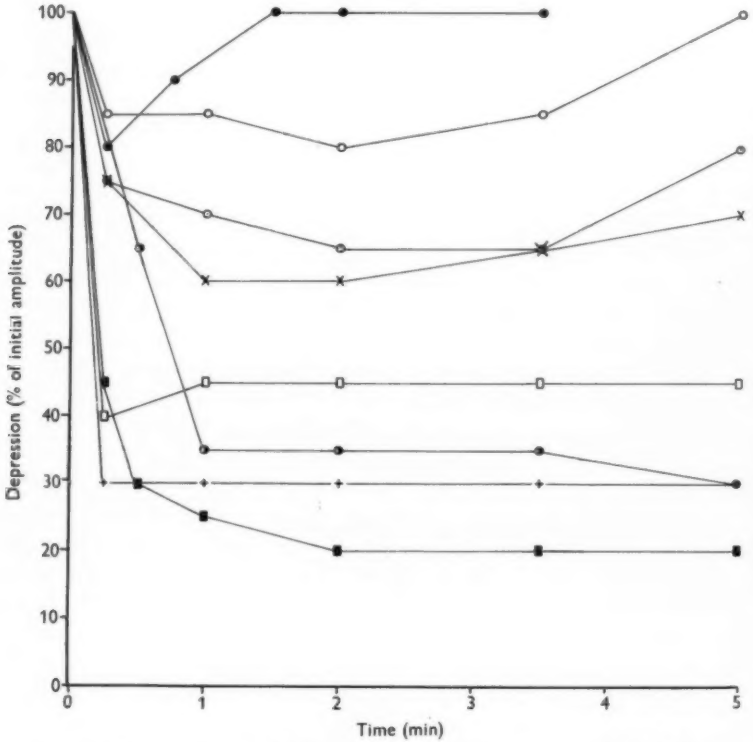

Fig. 2. Time course of the depression of the negative potential of the cortex (evoked by a nearby stimulus), brought about by topical application of the following solutions at zero time: (●) Control, and GABCh, 1 %; (○) GABA, 0·1 % (300 c.u.r./ml.); (⊙) Factor I Fraction B, 250 c.u.r./ml.; (×) BGPA and GGBA, 1 % (1000 c.u.r./ml.); (□) Factor I, fraction A, 240 c.u.r./ml.; (◑) GABA, 1 % (3000 c.u.r./ml.); (+) Factor I, fraction B, 750 c.u.r./ml.; (■) Factor I, fraction A, 720 c.u.r./ml.

during the course of the experiment. It was found that in any one experiment the degree of depression produced by a given solution was consistent, but that there was some variation between animals. Nevertheless, the same order of effectiveness was always observed; thus 1 % GABA always gave a greater degree of inhibition than did 1 % GCBA, but less than either

Factor 1 fraction (720 or 750 c.u.r./ml.), etc. These comments on variability apply also to the results on the other preparations described below.

Responses of a sensory pathway

Application of fraction B of Factor I to the nucleus gracilis caused, after a brief transient depression observed also with Locke's solution, a more prolonged inhibition in amplitude of the post-synaptic spike recorded from the nucleus and an associated reduction of the evoked potential of the sensory cortex (Fig. 3). These effects were always reversible even when the area to which the solutions were applied was not rinsed, and they

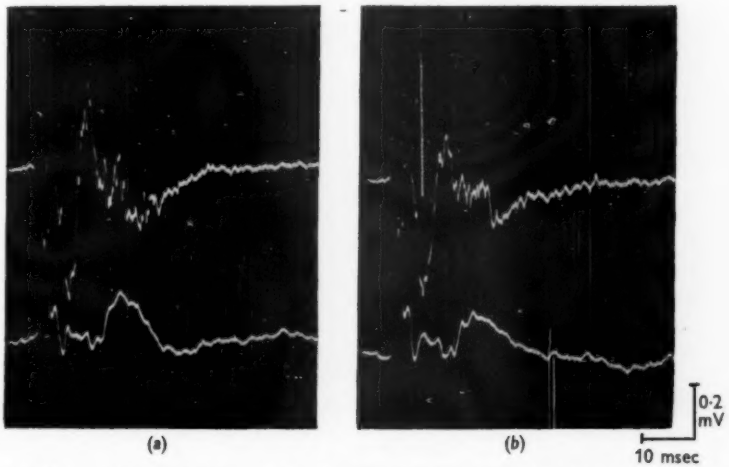

Fig. 3. Potentials recorded from the nucleus gracilis (upper trace) and contralateral sensory cortex (lower trace). (*a*) Control; (*b*) 1 min after application of 2 drops of Factor I, fraction B (500 c.u.r./ml.) to the nucleus. The incoming presynaptic spike in the nucleus is not shown in these records, being too large to appear at the gain used.

were not associated with any change in amplitude of the presynaptic spike which is also recorded from the nucleus. The observed depressions would therefore appear to be due to changes in the excitability of the post-synaptic cells in the nucleus, and the alteration in the cortical response is a direct consequence of this depression.

Both Factor I fractions, GABA, BGPA and GGBA were all able to produce these effects, and characteristic results are illustrated in Fig. 4*a* and *b* (not from the same experiment as Fig. 3). In roughly equivalent concentrations (in terms of crayfish units) fraction B was considerably more active than fraction A, and none of the pure substances tested

approached it in potency when applied as 1% solutions. Again the 1% solution of GABCh was found to be inert on this structure.

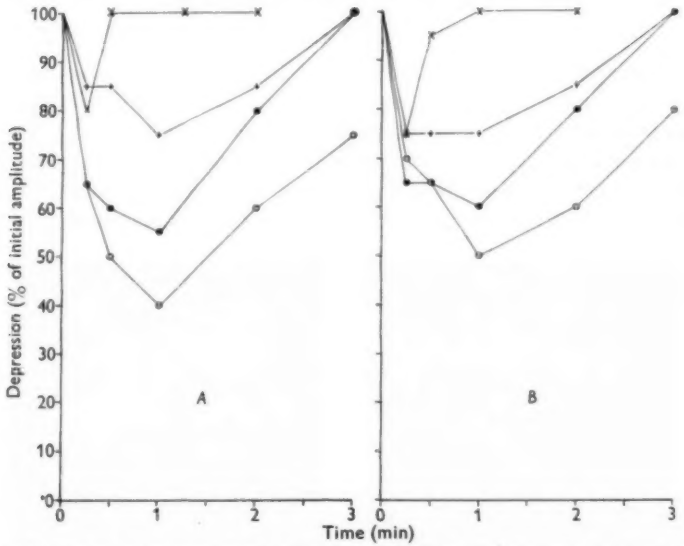

Fig. 4. Typical time courses of depression of (*A*) the post-synaptic spike of the nucleus gracilis and (*B*) of the associated evoked cortical potential, following application of the following solutions to the nucleus at zero time. (×) Control, and GABCh, 1%; (+) Factor I, fraction A, 720 c.u.r./ml.; GGBA, 1% and BGPA, 1%; (●) GABA, 1%; (⊙) Factor I fraction B, 750 c.u.r./ml.

Monosynaptic reflexes

The inhibitory effect of solutions of Factor I on the monosynaptic tendon jerk reflexes of the spinal cord, and the antagonism of this inhibitory effect by the prior administration of subconvulsive doses of strychnine to the animal, have been reported by Florey & McLennan (1955*b*). The failure of strong solutions of GABA to produce any detectable effect on this reflex pathway has been demonstrated (McLennan, 1957), and Curtis, Phillis & Watkins (1959) have shown that GABA has only a non-selective depressant action on the spinal motor neurones when it is applied to their cell somata electrophoretically. In the present experiments it was found that both fractions A and B of Factor I caused an immediate inhibition of the reflex extension of the cat's hind leg which can be elicited by electrical stimulation of a sensory root (Fig. 5*c* and *d*). The inhibition was readily reversed when the surface of the cord was washed with Locke's solution. Fig. 5*a* and *b* shows also that 1% solutions of GABA, GGBA and GABCh were entirely without effect.

The two Factor I fractions differed, however, in their actions after strychnine. After the I.V. administration of strychnine sulphate 0·05 mg/kg, application of fraction A no longer caused any inhibition of the reflex (Fig. 5*c*), while an equal concentration, in terms of crayfish activity, of

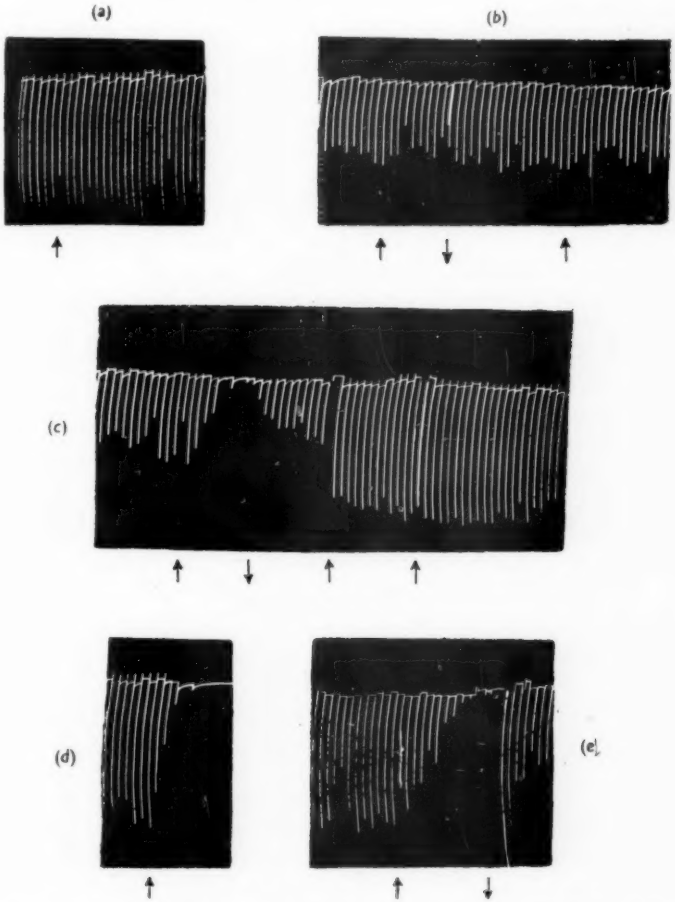

Fig. 5. Records of movements of the cat hind leg evoked by electrical stimulation of a dorsal rootlet, 1/10 sec. At ↑, the following solutions were applied to the cord: (*a*) GABA, 1%; (*b*) GGBA, 1%; wash; GABCh, 1%; (*c*) Factor I, fraction A, 300 c.u.r./ml.; wash; strychnine sulphate 0·05 mg/kg I.V.; repeat Factor I; (*d*) Factor I, fraction B, 300 c.u.r./ml.; (*e*) Fraction B after strychnine sulphate 0·08 mg/kg; wash. Note the post-inhibitory facilitation previously observed by Florey & McLennan (1955*b*).

fraction B (300 c.u.r./ml.) was still effective, although it was noted that the time required for the development of complete inhibition after strychnine was now prolonged (Fig. 5*e*).

Inferior mesenteric ganglion

It has been reported that the responses recorded from the post-ganglionic nerves leading from the inferior mesenteric ganglion of the rabbit *in vitro* are markedly inhibited by the topical application of Factor I solutions to the ganglion (Florey & McLennan, 1955*a*). This observation has been confirmed in the present series of experiments and Fig. 6 shows the time courses of depression of the response obtained in a typical experiment.

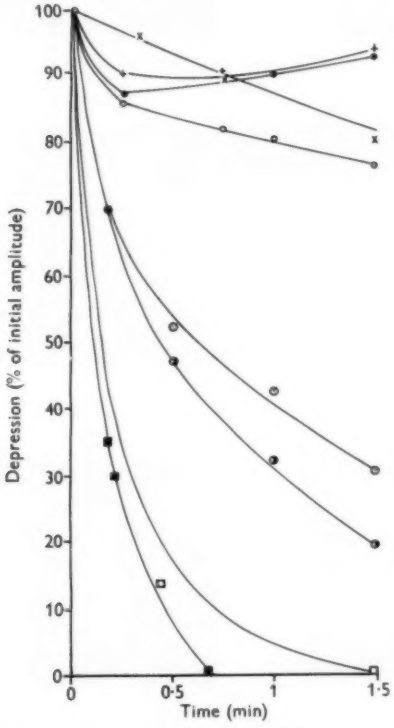

Fig. 6. Time courses of depression of the post-ganglionic response recorded in the ascending mesenteric nerve of the rabbit's inferior mesenteric ganglion. Solutions were applied to the ganglion at zero time. (+) Factor I, fraction A, 120 c.u.r./ml.; (●) GABA, 1 %; GGBA, 1 %, GABCh, 1 %; (×) Factor I, fraction B, 125 c.u.r./ml. (○) Factor I, fraction B, 150 c.u.r./ml.; (⊙) Factor I, fraction A, 180 c.u.r./ml.; (◑) Factor I, fraction B, 190 c.u.r./ml.; (□) Factor I, fraction B, 250 c.u.r./ml.; (■) Factor I, fraction A, 240 c.u.r./ml.

Both fractions of Factor I were able to bring about this effect, and equivalent concentrations expressed in terms of crayfish activity gave almost equal degrees of inhibition.

Of the various pure substances which have been tested for their effects on this structure none has been found to have powerful or sustained actions. As is shown in Fig. 6, the application of 1 % solutions of GABA, GGBA or GABCh resulted only in a small, transient depression of the post-ganglionic spike, a result which was obviously different from that observed after the application of Locke's solution. These small inhibitory effects were spontaneously reversible, but the more profound depressions observed with Factor I solutions were reversed only by rinsing the ganglion for 2–30 min.

DISCUSSION

The present experiments are in agreement with conclusions reached in earlier work regarding the non-identity of Factor I and GABA. It is clear that some biological preparations which have been used in the past in an attempt to characterize the active components in brain extracts are relatively non-specific, for example, the crayfish stretch receptor neurone. The present results indicate that the same is true for the responses evoked in the cerebral cortex by direct stimulation and those of the relay neurones of an afferent sensory pathway, although, in these cases too, GABA and Factor I have qualitatively similar effects, and although there is quantitative agreement when the effects are compared with those on the crayfish. Thus, for example, 1 c.u.r. of GABA assayed on the stretch receptor is 3 μg (McLennan, 1957, 1959) while for cortical inhibition 1 c.u.r. is equivalent to about 4 μg of GABA.

Investigation of the chromatographic characteristics of the active material in Factor I responsible for inhibition of the crayfish stretch receptor neurone discharge resulted in the demonstration that there are at least two active components, and that one of them might be an ω-guanidino acid, specifically either BGPA or GGBA (McLennan, 1959). These compounds were shown to be almost as active as GABA on the crayfish. In the light of the present results, however, it appears that these acids resemble GABA in their actions more than they do Factor I, for they act similarly on the cortical responses and have a similar lack of effect on monosynaptic reflexes in the cord and on transmission in a sympathetic ganglion.

Another compound which has been tested is GABCh, which has an effect on the stretch receptor and strongly antagonizes acetylcholine on the sea urchin oesophagus (McLennan, 1959). It has been reported that this ester has a potent effect on evoked cortical potentials (Takahashi *et al.* 1958), but this observation could not be confirmed in the course of the present

work even when concentrations ten times higher than those of Takahashi *et al.* were used. The results of the present experiments together with those reported earlier make it extremely unlikely that this compound is involved in Factor I action.

In contrast to the similarities observed between Factor I and some of the synthetic compounds in their action on the sensory pathway and on the directly evoked cortical potentials, marked differences were found with the other two preparations. Thus transmission processes through the inferior mesenteric ganglion of the rabbit and the monosynaptic reflex activity of the cat's spinal cord were strongly inhibited by Factor I, but little or not affected by the synthetic compounds. In conformity with earlier work, then, all the actions of Factor I cannot be explained in terms of GABA, and the same is true also for the guanidino acids. The identity of the compounds responsible for the effects of Factor I on the cord and on the ganglion remains unknown.

The observation that the action of Factor I solutions on monosynaptic reflexes is abolished by strychnine has been confirmed for fraction A of Factor I, whereas with fraction B only the time course of development of the inhibition was found to be prolonged. Part of the interest in Factor I lies in the possibility that it contains a transmitter substance active at inhibitory synapses, and Eccles (1957) has shown that subconvulsive doses of strychnine block the action of the physiological inhibitory pathway impinging upon the monosynaptic reflex arc. It is, therefore, reasonable to expect that strychnine might prevent the action of Factor I on the reflex also, if it does in fact contain an inhibitory transmitter substance. Eccles has further suggested that strychnine acts by a competitive inhibition with the inhibitory transmitter at the subsynaptic membrane, i.e. the concentrations of the drug and of the transmitter interacting will determine the final effect. It may be possible to explain the difference observed between fraction A and fraction B of Factor I in susceptibility to strychnine (on monosynaptic reflexes) on this basis. The suggestion is that fraction B may contain more material active on the cord than does fraction A, although the activities of the applied solutions towards the crayfish stretch receptor neurone are approximately equal. Thus the effect of applied fraction A might be completely prevented by strychnine, while that of B would be only delayed. It should be noted that the solutions of Factor I used in these experiments are stronger than those used earlier (Florey & McLennan, 1955*b*; McLennan, 1957).

SUMMARY

1. The effects of brain extracts containing a principle causing inhibition of the discharge of the crayfish stretch receptor neurone (Factor I), and of a number of pure compounds, have been compared on (*a*) directly evoked cortical potentials, (*b*) synapses in an afferent pathway, (*c*) a monosynaptic reflex arc, and (*d*) transmission in the inferior mesenteric ganglion.

2. The structures responsible for the directly evoked potentials in the cortex, and the synapses in the nucleus gracilis are depressed by topical application of Factor I, γ-aminobutyric acid, β-guanidinopropionic acid and γ-guanidinobutyric acid.

3. Monosynaptic stretch reflexes and transmission through the inferior mesenteric ganglion are blocked by Factor I, but not by the pure compounds used.

4. The activity of one fraction of Factor I is completely antagonized in the cord by strychnine, but that of the other is not.

5. γ-Aminobutyrylcholine has been found to be inactive on the four structures tested.

6. It is concluded that none of the pure substances tested can explain all the actions of Factor I.

We are much indebted to the Daiichi Seiyaku Pharmaceutical Co., Tokyo, for generous supplies of γ-aminobutyrylcholine. This work was supported by grants to one of us (H. McL.) from the National Research Council of Canada and the Leon and Thea Koerner Foundation.

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ELECTROPHYSIOLOGICAL EVIDENCE OF BARORECEPTORS IN THE PULMONARY ARTERY OF THE DOG

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There is evidence that reflex cardiovascular changes may be initiated by alterations in pulmonary arterial pressure (for references see p. 329); and endings, histologically similar to those in known baroreceptor areas, have been found in the pulmonary artery (e.g. Larsell & Dow, 1933; Takino, 1933; Nonidez, 1941). However, apart from the recording of impulses from a single receptor located in the pulmonary artery of a cat (Swan & Whitteridge, 1956), there has been no demonstration of the existence of such receptors by means of electrophysiological techniques.

In the work to be reported here impulse activity has been recorded in vagal fibres whose endings could be located with certainty in the pulmonary artery. In preliminary experiments on cats we were able to confirm the findings of Swan & Whitteridge (1956). The present account deals with experiments performed on dogs; for, technically, it is easier to locate receptors precisely in the larger pulmonary artery of the dog. A brief account of these findings has already been published (Coleridge & Kidd, 1959*a*).

METHODS

Experiments were performed on dogs (7·5–22·5 kg) anaesthetized with morphine sulphate (3 mg/kg subcutaneously) and 0·25 ml./kg (intravenously) of a 1 : 1 mixture of Dial Compound (allobarbitone-urethane, Ciba) and sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd.) solutions.

A tracheal cannula was inserted, and the lungs were ventilated by a Starling 'Ideal' pump. The sternum was split in the mid line, and the internal mammary vessels were ligated and cut. The cut edges of the sternum were retracted widely to give a good exposure of the heart and great vessels. The pericardium was opened in the mid line.

Respiration was recorded with an optical manometer (Coleridge & Linden, 1954) attached to a side arm on the tracheal cannula. In some experiments arterial blood pressure was also recorded optically from the ascending aorta with a cannula inserted through the left common carotid artery.

Action potentials were recorded from afferent single-fibre preparations dissected from the cervical vago-sympathetic trunk by the technique previously described (Coleridge, Hemingway, Holmes & Linden, 1957). The action potentials were amplified by a conventional R-C coupled amplifier and were displayed with an e.c.g. on a double-beam cathode ray tube. By suitable optical systems a combined photographic recording was obtained of the two

oscilloscope traces, the beams from the optical manometers, a 50 c/s time trace and a signal marker.

In some experiments infusions of saline (NaCl solution 0·9 % (w/v)) or dextran (6 % (w/v) in NaCl solution 0·9 % (w/v); 'Dextraven', Benger Laboratories Ltd.) at body temperature were made into the femoral vein.

The site of a receptor, from which action potentials were being recorded, was explored first by temporary obstruction, in turn, of the main intrathoracic vessels. For this purpose soft string ligatures were placed round the origin of the main pulmonary artery, both lung roots and the ascending aorta; the ends of the ligatures were passed through short lengths of polythene tube to form snares. Further location was effected by distending the pulmonary artery with a balloon. This device, modified from the self-guiding catheter described by Lategola & Rahn (1953), consisted of two polyvinyl tubes (approximately 70 cm long, bore 1·0 mm) cemented side by side. One tube served to distend the balloon which consisted of a condom teat fastened by thread. The other tube, the open tip of which lay immediately proximal to the balloon, was connected through a 3-way stop-cock either to a heparinized saline drip (Pularin Evans 5000 i.u./l., in NaCl solution 0·9 % (w/v)) or to a saline manometer. The collapsed balloon was inserted into either external jugular vein. Approximately 1 ml. saline was injected into the balloon; then the catheter was pushed gently into the vein and the partially distended balloon was carried into the right heart by the blood. Its passage from atrium into ventricle and subsequently into the pulmonary artery was indicated by the pressure registered by the saline manometer. Once placed in the main pulmonary artery, the balloon was collapsed completely until required.

Finally, the position of a receptor was determined accurately by careful exploration of the pulmonary artery with a fine probe immediately after the animal had been killed and the heart and great vessels dissected.

RESULTS

In twenty-nine instances impulses recorded from a slip of the vagus nerve were found to originate from receptors in the pulmonary artery between the main bifurcation and the lung roots. The discharges in these fibres were affected in characteristic ways by various experimental procedures. These are now described in the order in which they were usually applied in the course of locating a receptor.

Identification of pulmonary arterial fibres

All pulmonary arterial receptors displayed a cardiac rhythm (e.g. Fig. 1), usually very similar to that of aortic baroreceptors (e.g. Fig. 3) in that the main discharge began in ventricular systole soon after the opening of the pulmonary and aortic valves, as judged by the relation of the discharge to the QRS complex of the electrocardiogram.

Effect of occluding the main pulmonary artery. Occlusion of the main pulmonary artery near its junction with the right ventricle invariably reduced or abolished the discharge from a pulmonary arterial receptor (Fig. 2). Clearly this receptor was not in the right atrium or ventricle but lay beyond the occluding ligature. The pattern of discharge during the first 4–5 heart beats after release of the vessel was similar to that of an aortic baroreceptor. But in Fig. 2 it will be seen that the discharge

disappeared and reappeared with the first heart beat after obstructing and releasing the pulmonary artery; moreover, the impulse activity attained its maximum frequency in the first 1–2 beats after release of the ligature, thereafter the frequency rapidly diminished. Its behaviour thus differed

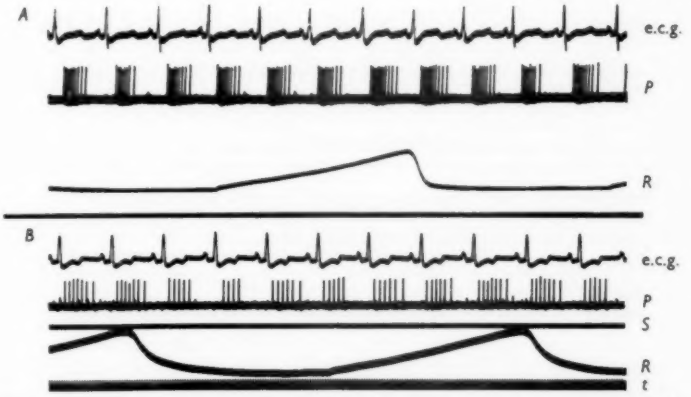

Fig. 1. Fibres from two receptors in the pulmonary artery showing burst of impulses during ventricular systole. In this and subsequent figures the following abbreviations are used: e.c.g., electrocardiogram; *P*, action potentials from vagal slip; *t*, time trace (1/50 sec); *R*, tracheal pressure (upstroke representing inflation); *S*, signal marker.

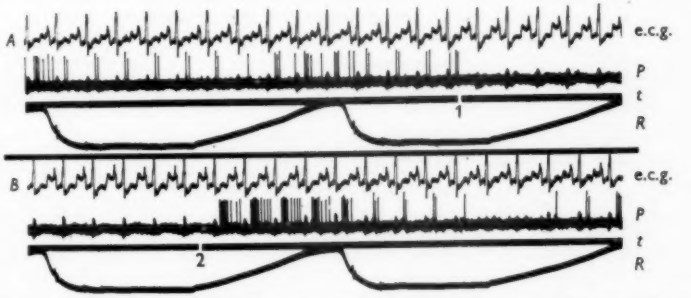

Fig. 2. Effect of occluding main pulmonary artery on discharge from pulmonary arterial receptor. Records *A* and *B* are continuous. At 1 the ligature around the proximal part of the main pulmonary artery was tightened; the ligature was released at 2.

from that of an aortic baroreceptor, as shown in Fig. 3. With aortic receptors there was always a delay of several beats before occlusion and release of the pulmonary artery affected the discharge.

These effects are in accordance with the results of other experiments in which pulmonary and systemic arterial pressures were measured

simultaneously. Occlusion and release of the pulmonary artery produced alterations in arterial pressure on the right and left sides of the heart whose time course was similar to that followed by the changes in impulse frequency recorded from the corresponding arterial receptors.

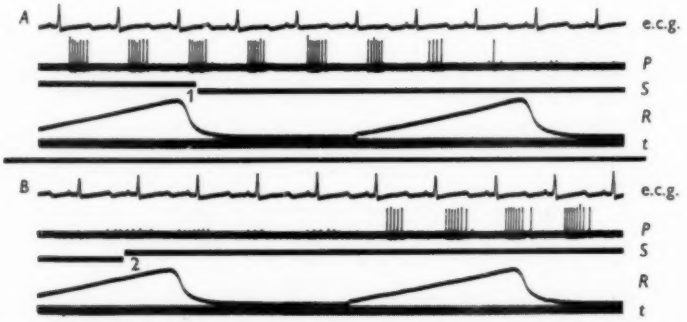

Fig. 3. Effect of occluding main pulmonary artery on discharge from an aortic baroreceptor. Interval of approximately 1 sec between records *A* and *B*. At 1 the ligature around the pulmonary artery was tightened; at 2 the artery was released.

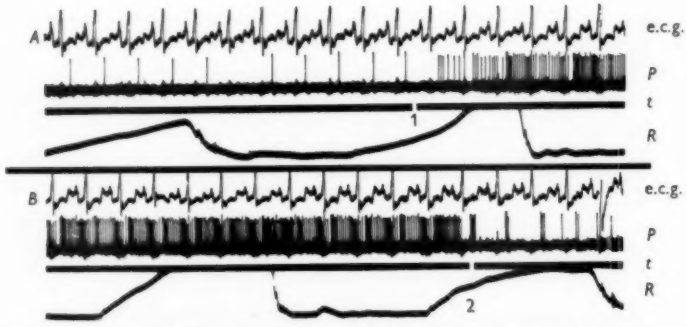

Fig. 4. Effect of occluding lung roots on discharge from receptor in pulmonary artery; same fibre as in Fig. 2. Records *A* and *B* are continuous. At 1 the snares around both right and left lung roots were tightened (note increase in tracheal pressure during period of occlusion); the snares were released at 2.

Effect of occluding the lung roots. All fibres whose impulse activity was not reduced or abolished by occlusion of the main pulmonary artery were discarded. Remaining fibres were then investigated by tightening the snares placed round the lung roots and observing the response to the subsequent increase in pulmonary arterial pressure. The effects thus produced on the discharge from a receptor in the pulmonary artery are demonstrated by the records shown in Fig. 4. Although greatly increased

by occlusion of the lung roots, the discharge was still discontinuous and showed a cardiac rhythm. By contrast, compressing the lung roots abolished the discharge from an aortic baroreceptor, as in Fig. 5. Since both pulmonary arteries and veins were included in the lung-root ligatures, there was less delay before occlusion abolished the discharge from this receptor than had been the case when the main pulmonary artery alone was occluded (Fig. 3). From the results depicted in Figs. 2 and 4, it was clear that the receptor was situated in the pulmonary artery between the conus and the snares around the lung roots.

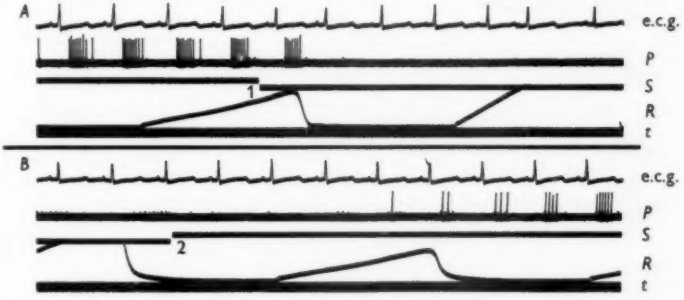

Fig. 5. Effect of occluding lung roots on discharge from an aortic baroreceptor; same fibre as in Fig. 3. Interval of approximately 2 sec between records *A* and *B*. At 1 the snares around both right and left roots were tightened (note increase in tracheal pressure during occlusion); at 2 the snares were released.

Effect of distending a balloon in the pulmonary artery. External palpation of the intact artery was of little value in locating a receptor, because much of the vessel was obscured by adjacent structures. Some dissection, with the inevitable risk of damage to nerves, was necessary. But the chances of retaining an active fibre were increased when the approximate site of the receptor was first determined in the intact vessel by distension of the balloon.

When the balloon was placed in the main artery proximal to the bifurcation, distension always reduced or abolished the discharge; hence all the receptors were apparently at or beyond the bifurcation. Distension of the balloon in the right or the left pulmonary artery produced two main types of result. Thus, distension of a balloon placed in the left artery invariably led to an increase in the impulse frequency of a receptor in the right artery (Fig. 6). This was in accordance with the observation that obstruction of flow to one lung causes an increase in pressure in the main pulmonary artery (e.g. Carlens, Hanson & Nordenström, 1951). On the other hand, when balloon and receptor were in the same branch the response to distension varied with their relative positions. Sometimes the

discharge just disappeared, when the receptor was distal to the balloon. On other occasions, distension of a balloon in the vicinity of a receptor caused an initial increase in the discharge, but with further distension the discharge was abolished, to return only when the balloon was finally collapsed (Fig. 7). This might have been due to the further distension displacing the balloon so that it moved proximal to the receptor.

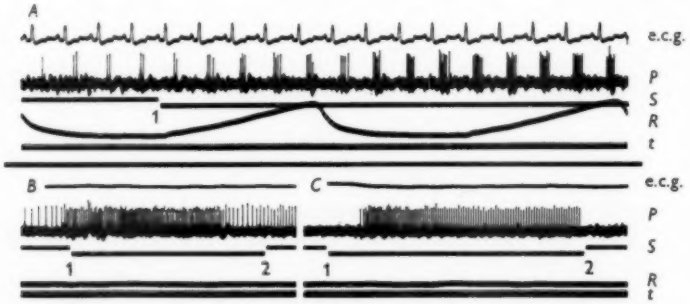

Fig. 6. Stages in the location of a pulmonary arterial receptor. *A*, inflation of balloon previously inserted into the left branch of the pulmonary artery; inflation started at 1. The obstruction of flow to the left lung produced an increase in the frequency of discharge from the receptor which was subsequently found to be situated in the right pulmonary artery. The animal was then killed, and the pulmonary artery opened. *B* and *C* show the discharge produced by punctate pressure (between 1 and 2) on the right pulmonary artery.

Punctate location of receptors. The animal was killed and the right ventricle and pulmonary conus were opened. The balloon was left *in situ* to serve as a guide. The artery was opened gradually and the interior carefully probed with a fine glass rod to find the point from which a high-frequency discharge could most easily be elicited (Figs. 6, 8). It was essential to see clearly the region stimulated by the probe. To this end it was often necessary to dissect the ascending aorta and the superior vena cava (see Fig. 9), and in many cases the resultant damage to nervous structures caused action potentials to disappear before a receptor had been located precisely.

Patterns of discharge in pulmonary arterial fibres

To determine accurately the distribution of the endings in the pulmonary artery it was necessary to be able to move the balloon freely within the artery and to have ready access to the heart and great vessels. Consequently, no attempt was made to record pulmonary arterial pressure. Even so, the evidence suggested that in general the endings responded to the pressure within the pulmonary artery. Thus, the ventricular systolic

pattern of discharge seen in the majority of these fibres was similar to that recorded from aortic baroreceptor fibres (compare Figs. 1 and 3), and corresponded in time to the expected pressure variations in the pulmonary artery. Again, the changes in impulse frequency on occlusion of the pulmonary artery and lung roots were in the direction in which the pulmonary arterial pressure undoubtedly changed.

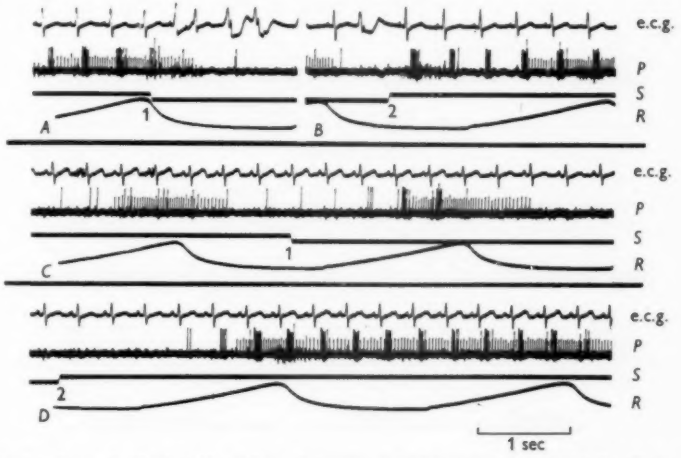

Fig. 7. Stages in the location of a pulmonary arterial receptor. *A* and *B*, effect of occlusion (at 1) and release (at 2) of main pulmonary trunk. Interval of approximately 4 sec between records *A* and *B*. *C* and *D*, alterations in the impulse activity produced by distension of a balloon in the vicinity of the receptor in the left pulmonary artery. Interval of 2 sec between records *C* and *D*. Distension of balloon started at 1, deflation at 2. Note also pulmonary stretch fibre (smaller spikes) which shows increase in discharge on deflation of balloon. In *A* and *C* some of the spikes have been retouched to aid reproduction.

However, unexpected patterns of discharge were found. For example, in the record shown in Fig. 2 there were occasional impulses during the P-R interval (i.e. synchronous with atrial systole), in addition to the main discharge starting in ventricular systole. In some instances the discharge during ventricular diastole was the most prominent. The pattern of discharge recorded when one fibre was first placed on the electrodes is shown in Fig. 8*A*; the discrete burst of activity starting in the P-R interval might well have been recorded from a type *A* receptor in the atrium or great veins (Paintal, 1953). However, infusion of 200 ml. dextran solution intravenously produced a marked alteration in the discharge (Fig. 8*B*); and although impulses synchronous with the P-R interval soon returned after the end of the infusion, main activity now occurred during ventricular systole. Consequently, it seemed worth while to try the effects of occluding

the main pulmonary artery (Fig. 8*C*). The abrupt return of a high-frequency discharge in the cardiac cycle immediately after release of the ligature suggested that this receptor was probably in the pulmonary artery. This was confirmed by occluding the lung roots. The atrial systolic discharge was thought to be due to distortion caused by atrial contraction; for all endings showing such a discharge were found, when located precisely, to be situated in the right branch of the pulmonary artery behind the superior vena cava (Fig. 8*D*).

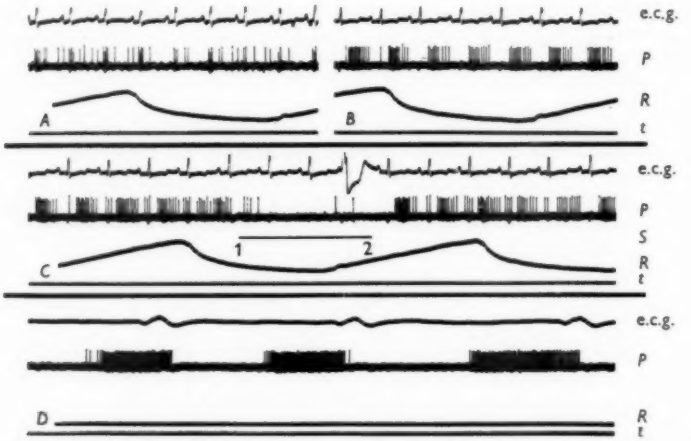

Fig. 8. Stages in the location of a pulmonary arterial receptor. *A*, pattern of discharge recorded when the fibre was first placed on electrodes. *B*, after the infusion of 200 ml. dextran solution intravenously. *C*, effect of (1) occlusion and (2) release of the main pulmonary artery. *D* shows the discharge produced by three punctate stimulations of the interior of the right branch of the pulmonary artery lying behind the superior vena cava after the animal had been killed, the pulmonary artery opened and the superior vena cava cut away.

Thus the pattern of discharge was affected not only by the degree of distension of the pulmonary artery but also by the position of the receptor in the artery. These results emphasize that a receptor cannot be assigned with certainty to a particular great vessel or chamber of the heart by inspection of the discharge and its relationship to the e.c.g.; it must be located by appropriate means in the animal with open chest. Such factors may have contributed to the previous paucity of information about receptors in the pulmonary artery.

Position of receptors in pulmonary artery

Recordings were made from twenty-nine fibres whose endings were situated in the pulmonary artery between the ligatures at the conus and

those around the lung roots. In eighteen it was not possible to determine the position of the receptor more precisely than this; for impulse activity disappeared before complete location had been achieved; but in the remainder the position of the receptor was determined precisely, as depicted in Fig. 9. All these eleven receptors were found in the vicinity of the main bifurcation, or in the right and left branches of the pulmonary artery between the bifurcation and the origins of the lobar branches.

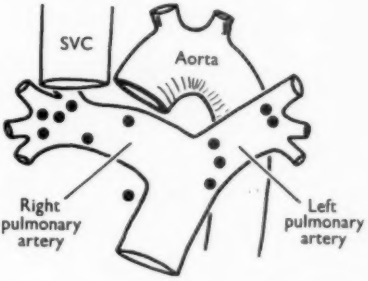

Fig. 9. The position of eleven pulmonary arterial receptors whose location had been determined accurately by punctate stimulation after the animals had been killed and the pulmonary artery dissected. The ascending aorta and the superior vena cava (SVC) near its junction with the right atrium which lie anterior to the right pulmonary artery have been omitted for clarity. In addition note the single receptor on the right of the main artery; this was situated in connective tissue between the pulmonary trunk and the first part of the ascending aorta.

Although some nerves were undoubtedly damaged in the final dissection of the pulmonary artery, our failure to find any receptors in the main pulmonary trunk proximal to the bifurcation cannot be thus explained; for the main artery was never opened until it had first been explored with a probe inserted through the right ventricle. Moreover, in the twenty-nine fibres, distension of a balloon in the main pulmonary artery never caused an increase in impulse frequency.

An additional receptor was found in connective tissue between the main pulmonary trunk and the ascending aorta (Fig. 9). The alterations in impulse frequency on occlusion of different vessels had led us to believe that this receptor was situated in the wall of the pulmonary artery. It was approximately in the location of the paraganglion aorticum supracardiale (Penitschka, 1931; group 4 of the aortic bodies described by Howe, 1956). However, at present we are unable to assess the significance of this single receptor.

In early experiments single-fibre preparations were dissected from either right or left vagus nerves. But, because only one pulmonary arterial

fibre was found in the right vagus in these first few dissections, we subsequently recorded from the left vagus. This obviously precludes comment on the relative incidence of pulmonary arterial fibres in the two nerves. Nevertheless, we do not think that this probably premature exclusion of the right vagus from our investigation significantly affected the findings as to the apparent distribution of receptors within the pulmonary artery, because the left vagus carried fibres from both right and left arterial branches.

DISCUSSION

The present investigation has shown that the vagus nerves of the dog contain afferent fibres whose endings are situated in the walls of the pulmonary artery in the region of the main bifurcation and in the extrapulmonary portions of the right and left branches, but not in the main pulmonary trunk proximal to its bifurcation. In this respect our findings are in agreement with most histological observations.

Nettleship (1936) described a sensory plexus on the base of the pulmonary artery in the cat; and Boyd (1941) reported a few scattered endings of the baroreceptor type in the distal part of the pulmonary arterial trunk and in the proximal part of the right and left branches in the rabbit. Otherwise histological evidence of baroreceptors in the pulmonary trunk proximal to the main bifurcation is lacking. The endings described by Larsell (1921) and Larsell & Dow (1933) in the rabbit and man, respectively, were limited to the pulmonary arteries near the hilum of the lung. Nonidez (1935, 1941) stated that although in the guinea-pig, rabbit and cat endings of the baroreceptor type were to be found in the arterial ligament, there was a complete absence of receptors in the wall of the pulmonary trunk; and apart from some endings in the right artery in one kitten, the proximal parts of the main branches were equally devoid of receptors. In the dog, on the other hand, Nonidez (1941) found that the endings extended beyond the arterial ligament to form a baroreceptor area over the anterior surface of the pulmonary arterial bifurcation. The absence of receptors in the main pulmonary trunk of the rabbit, cat, dog and man was confirmed by Takino (1933) and Takino & Watanabe (1937). But in contrast to the findings of Nonidez, these workers described a wide distribution of baroreceptor endings on the right and left branches extending between the main bifurcation and the lung roots.

It seems that the receptor area is not limited to the extrapulmonary vessels examined in the present investigation; for similar endings have also been demonstrated histologically in the intrapulmonary branches of the artery (Dogiel, 1898; Larsell, 1921, 1922; Larsell & Dow, 1933; Takino, 1933; Takino & Watanabe, 1937). Furthermore, impulse activity with a similar ventricular systolic discharge has been recorded in fibres arising

from what were believed to be vascular receptors in the lungs (Coleridge & Kidd, 1959*b*). But it is not possible with electrophysiological methods to locate such intrapulmonary endings to a particular vessel, and they have been excluded from the present account which deals only with receptors whose situation could be determined precisely.

In the present experiments many more aortic baroreceptor fibres were encountered in the vagus of the dog than fibres from the pulmonary artery. But fibres could be identified only when they were active; and in view of the sparse activity exhibited by some fibres (e.g. Fig. 4), it is possible that other pulmonary arterial fibres were completely inactive under the conditions of our experiments. The prolonged exposure of the heart with repeated occlusion of the great vessels undoubtedly caused some cardiac deterioration. The right ventricle was affected more than the left by these adverse conditions because the main pulmonary artery was the vessel most commonly occluded. And since pulmonary baroreceptor discharge was dependent upon the pressure developed in the pulmonary artery by right ventricular contraction, this may have accounted in part for our impression of the relative paucity of pulmonary as compared with aortic baroreceptor fibres. However, the histological evidence also suggests that, in contrast to the rich innervation of the arch of the aorta, baroreceptor endings are distributed quite sparsely in the wall of the pulmonary artery (Takino & Watanabe, 1937; Boyd, 1941; Nonidez, 1941). This raises the question of the functional significance of the pulmonary arterial receptors.

Much evidence indicates that reflex changes can be elicited from the lesser circulation. Thus, cardiovascular and respiratory effects, resulting from stimulation of receptors in the lungs, can be elicited by injection of various chemical substances (reviewed by Dawes & Comroe, 1954). It has also been shown that a large increase of vascular pressure in a perfused lung may be followed by a reflex fall in systemic arterial pressure, bradycardia and variable changes in respiration (Churchill & Cope, 1929; Harrison, Calhoun, Cullen, Wilkins & Pilcher, 1932; Schwiegk, 1935; Schweitzer, 1936; Parin, 1947); but it was concluded that the receptors mediating these effects were mainly on the venous side of the pulmonary vascular bed (Daly, Ludány, Todd & Verney, 1937; Downing, 1957).

On the other hand, Aviado, Li, Kalow, Schmidt, Turnbull, Peskin, Hess & Weiss (1951) reported that the receptors responsible for the bradycardia were in the pulmonary arterial trunk, although they agreed with previous workers that the sensory endings concerned in the hypotensive and respiratory changes were probably situated in the pulmonary veins. Takino & Watanabe (1937) described a reflex fall in systemic arterial pressure, and bradycardia, in response to electrical and mechanical stimulation of the pulmonary artery of the rabbit. It has also been suggested that

receptors in the proximal part of the pulmonary artery may mediate the 'triad' of hypotension, bradycardia and apnoea which follows injection of capsaicine in the dog (Pórszász, Such & Pórszász-Gibiszer, 1957). Clearly such effects could be explained by the receptors now described, but their normal function remains quite unknown.

SUMMARY

1. Electrophysiological techniques have been employed in anaesthetized dogs to demonstrate baroreceptor endings in the pulmonary artery.

2. Afferent impulses were recorded from single-fibre preparations dissected from slips of the cervical vagus. Receptors in the pulmonary artery were first located approximately by tightening ligatures placed around the origin of the pulmonary artery and around the lung roots. Further location was effected by distension of a balloon passed into the pulmonary artery from the external jugular vein. Finally, the position of a receptor was defined accurately by careful exploration of the pulmonary artery with a fine probe after the animal had been killed and the heart and great vessels dissected.

3. All receptors so located were found in the vicinity of the main bifurcation of the pulmonary artery, or in the right and left branches between the main bifurcation and the origins of the lobar branches. No receptors have so far been found in the pulmonary trunk proximal to the main bifurcation.

4. Recordings made from several fibres whose endings were located in the pulmonary artery showed a ventricular systolic pattern of discharge similar to that recorded from aortic baroreceptor fibres. Other fibres showed unexpected patterns of discharge in addition.

While this manuscript was being prepared we were informed by Dr R. Bianconi and Dr J. H. Green that they have recently employed electrophysiological techniques to investigate pulmonary arterial baroreceptors in the cat (Bianconi & Green, 1959). We are indebted to Professor A. Hemingway and Dr W. J. O'Connor for helpful criticism of the manuscript. We also wish to thank Miss Barbara Thomas and Mr S. Stainthorpe for technical assistance.

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THE EFFECT ON A MUSCLE TWITCH OF THE BACK-RESPONSE OF ITS MOTOR NERVE FIBRES

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When a muscle is activated by a single maximal shock applied to its nerve the resulting twitch may differ markedly from that produced by slightly asynchronous activation of the same muscle fibres. This surprising effect was discovered by Merton (1951, 1954), who showed that a 'synchronous' twitch is larger and lasts longer than a 'desynchronized' twitch, produced by splitting a maximal nerve volley into two half-volleys separated in time. On altering the time interval between the two half-volleys Merton found that the desynchronized twitch only began to become larger when the volleys were within 0·8 msec of each other, and he concluded that this effect of synchronization could not depend upon purely mechanical factors, such as those studied by Rushton (1932). He suggested instead that the propagation of the action potential along the different fibres was slowed when they were activated synchronously, and that this caused a prolongation of their 'active state'. The difference between the synchronous and asynchronous twitches could, however, also be explained by supposing that the synchronous activation produces a brief tetanic contraction of some muscle fibres. This might indeed be expected to occur, for the summed action potentials of the muscle fibres may excite the motor nerve fibres (Lloyd, 1942; Leksell, 1945) and this ephaptically induced 'back-response' of the nerve fibres might be expected to re-excite the muscle fibres. The present paper and a preliminary communication (Brown & Matthews, 1959) show that this does happen and that the differences in the back-responses elicited by synchronized and desynchronized activations of the muscle explain the differences between the tensions developed in the two cases.

METHODS

The experiments were performed on cats. Fifteen cats were lightly anaesthetized with pentobarbitone sodium (Nembutal; Abbott Laboratories) given intraperitoneally, and one was decerebrated. The soleus muscle and the medial head of the gastrocnemius muscle were studied, usually in separate experiments. Figure 1 shows the general arrangement of

* M.R.C. Scholar.

the experiment used in each case. The muscle was attached to an isometric myograph, the output of which was displayed on one cathode-ray tube. The soleus muscle was dissected free from gastrocnemius, but the medial head of gastrocnemius was not separated from the lateral head of the muscle. The muscle nerve was dissected free from other nerve branches for several centimetres central to the muscle, and most other limb nerves were cut. The nerve was stimulated electrically by a square-wave stimulator which delivered pulses of about 100 μsec duration. The compound action potential so excited in the motor fibres was recorded from the peripheral end of an appropriate ventral root and displayed on a second cathode-ray tube with a different sweep speed from that used to record the muscle contraction. Both cathode-ray tubes could be photographed simultaneously. In some experiments the electromyogram was also recorded on one of the cathode-ray tubes. Exposed muscles, nerves and spinal roots were covered in liquid paraffin maintained at body temperature and initially equilibrated with 95 % O_2, 5 % CO_2. Further details of the apparatus have already been described (Matthews, 1959).

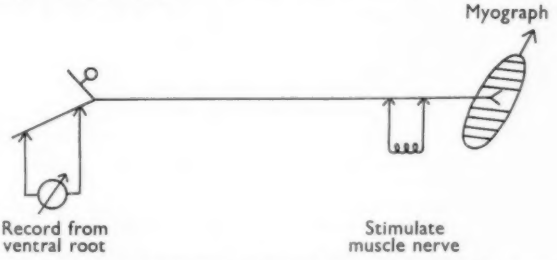

Fig. 1. The experimental arrangement used for simultaneously recording the contraction of the muscle and the back-response of its motor fibres.

RESULTS

Figure 2 *a* shows a typical record obtained from the S1 ventral root on stimulating the nerve to the medial head of gastrocnemius with a single shock. Following the stimulus artifact by 1·1 msec was the direct response of the α motor nerve fibres to the electrical stimulus; 1·8 msec later still there was an additional wave, which would be unusually large to be the response of the γ motor fibres (cf. Leksell, 1945; Matthews & Rushworth, 1957) and which was abolished when the nerve was crushed between the stimulating electrodes and the muscle (Fig. 2*d*). It must therefore have been initiated by the activity of the muscle. Leksell (1945) called this wave the 'back-response' and agreed with Lloyd (1941, 1942) in concluding that it was set up by ephaptic stimulation of the α motor fibres by the summed action potentials of the muscle fibres. In the present experiment it was regularly shown to be conducted in the α motor fibres, for it could be abolished by rendering these fibres refractory by exciting them with an appropriately timed second shock.

As a single stimulus leads to both the direct response and the back-response in the nerve it is reasonable to suggest that the muscle is thereby also doubly excited. This will occur if the interval between the direct

response and the back-response is greater than the refractory period of the muscle, as tested by two shocks applied to its nerve. Figure 2*b* shows results obtained when the stimulus interval was 1·35 msec, which was the minimum required to produce a marked increase in the size of the twitch (see also Fig. 8). Figure 2*c* shows the response of Fig. 2*b* superimposed on that of Fig. 2*a*, and confirms that the refractory period of the muscle was less than the time interval between the direct response and the back-response. The recorded interval between the direct response (D.R.) and the back-response (B.R.) should not, however, be compared directly with the refractory period of the muscle. For in the region of nerve between the stimulating electrodes and the muscle this D.R.–B.R. interval pro-

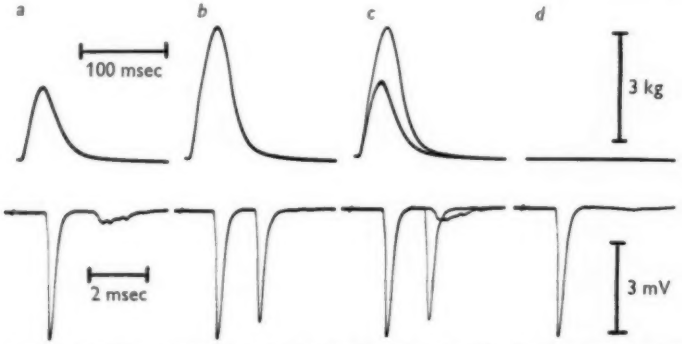

Fig. 2. Comparison of the latency of the back-response with that of a second action potential which was late enough to re-excite the muscle. Simultaneously obtained records of the contraction of the medial head of gastrocnemius (above), and of the action potential of its motor fibres (below). *a*, *b*, *c*, muscle nerve intact; *a*, single maximal shock to nerve giving a direct response followed by a back-response; *b*, two maximal shocks, the second occurring just late enough to re-excite the muscle; *c*, the responses of *a* and *b* superimposed; *d*, single shock, nerve crushed between the stimulating electrodes and the muscle in order to abolish the back-response. (All records in this and later figures consist of 6 superimposed sweeps. The conduction distance from the stimulating cathode to the muscle was 0·7 cm and to the recording electrode was 17·0 cm. Spikes retouched.)

gressively decreases, because the action potentials are there travelling in opposite directions. The minimum D.R.–B.R. interval must occur at the place on the nerve, somewhere in the muscle, where the back-response is initiated. It may be determined to a first approximation by subtracting from the observed interval twice the calculated conduction time from the stimulating electrodes to the muscle (estimated from the conduction velocity from the stimulating electrodes to the ventral root). In the experiment of Fig. 2 this correction was only 0·1 msec and the minimum B.R.–D.R. is therefore 1·7 msec, which was appreciably longer than the refractory period

of 1·35 msec. In this case, therefore, the back-response might have been expected to have re-excited the muscle fibres, and this has been the usual result on making such a comparison.

Unfortunately the estimate of the minimum D.R.–B.R. interval was necessarily unreliable, because the determination of the conduction time from the stimulating electrodes to the site of initiation of the back-response was liable to two uncertainties. First, the precise point of initiation of the back-response was unknown, and the present calculations have been made as if it were initiated at the point where the nerve entered the muscle. Secondly, when the nerve enters the muscle its fibres subdivide into thinner branches, which would be expected to conduct more slowly than the fibres within the main trunk. Thus it is probable that the minimum D.R.–B.R. interval has been systematically underestimated in the present experiments. If this error has been at all large ($> 0{\cdot}4$ msec) then a part of all of the back-response may not have re-excited the muscle fibres, because they were still refractory. Moreover, if, because of refractoriness of the nerve, the direct response set up by the second stimulus travelled more slowly than did that set up by the first stimulus, then the stimulus interval would be less than the interval between the time of arrival of the two action potentials at the nerve endings, and the refractory period would have been underestimated. There was not, however, appreciable slowing of the second action potential in the region of nerve between the stimulating and the recording electrodes, and the results of Eccles & O'Connor (1939) suggest that when the second stimulus fell outside the refractory period of the muscle such slowing did not occur in the nerve terminals within the muscle. Even if the initial part of the back-response failed to excite the muscle, it is possible that the later part of the back-response did so. For on recording from the ventral root the back-response lasted from 1·1 to 2·7 msec in different experiments, and this scatter probably resulted from the back-response being initiated at different times in different nerve fibres. (Differences of conduction velocity between different nerve fibres would not make the scatter of the back-response much greater than that of the direct response, unless the nerve terminations were behaving in a manner very different from the main nerve fibre.) Thus though the experiments so far described by no means prove that the back-response re-excites the muscle fibres, this does indeed appear probable.

Lloyd (1942) calculated that the earliest part of the back-response was set up within 0·1 msec of the beginning of the muscle action potential. Such a short interval might not allow time for the muscle fibres to recover from their refractory period before they were restimulated by the back-response. This very short interval was, however, found only in those nerve fibres which had not been excited by the initial electrical stimulus, which was applied to a ventral root and not to the muscle nerve. In those fibres which had been directly excited the back-response began significantly later, and Lloyd gives one example (Fig. 1) where the back-response in the directly stimulated nerve fibres began 0·9 msec after that of the unstimulated nerve fibres. In some of the present experiments the electromyogram was recorded with belly–tendon leads, and its latency could then be compared with the time of initiation of the back-response (calculated from the time of its appearance in the ventral root). In gastrocnemius the back-response was set up 0·6–1·1 msec after the beginning of the electromyogram (five experiments) and in soleus the interval was slightly longer (1·4 and 1·9 msec in the two experiments studied in this respect). Thus the delay between the back-response and the beginning of the electromyogram does not appear to be so short that re-excitation of the muscle by the back-response is impossible.

The effect of desynchronization

The abnormally synchronous nerve volley initiated by a single stimulus probably greatly favours the production of the back-response, for Lloyd

(1942, Fig. 6) found that the activity of different muscle fibres may sum to produce a back-response, and that this interaction only persisted for about 0·8 msec. Thus a slightly 'desynchronized' nerve volley might be expected to produce a smaller back-response than does a synchronized nerve volley, and in the present experiments this has been found to be the case. The usual method employed to desynchronize the action potential has been to apply two stimuli to the same point on the nerve and to separate them by a time interval just less than the refractory period of the nerve fibres. The first stimulus was adjusted so as to excite about half the

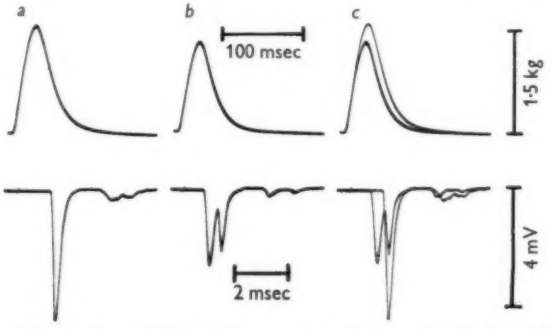

Fig. 3. The effect of desynchronizing the nerve volley in reducing both the muscle contraction (above) and the back-response (below). *a*, synchronous nerve volley produced by single maximal shock to nerve; *b*, desynchronized nerve volley produced by a submaximal shock followed by a maximal shock, falling within the refractory period of those nerve fibres excited by the submaximal shock; *c*, superimposed responses to synchronized and to desynchronized nerve volleys. (Same experiment as Fig. 2. Spikes retouched.)

α motor fibres, and the second was supramaximal (about twice the strength of that required to excite all the α motor fibres when delivered alone). Thus those fibres which were not excited by the first shock were excited by the second stimulus, and no fibres were excited twice; in consequence the single maximal volley which would have been produced by the second stimulus was split into two half-volleys separated by about 0·6 msec. Figure 3 shows such synchronized and desynchronized nerve volleys and demonstrates the typical result, that the desynchronized volley caused appreciably less back-response. In four experiments in which the back-response was small it was abolished by desynchronizing the nerve volley. Figure 3 also shows that the twitches produced by the two types of nerve volley differed markedly. The desynchronized twitch was smaller and decayed more rapidly than the synchronized twitch, as has already been described by Merton (1954). These are the changes which would be expected if the back-response were exciting the muscle, for the

synchronized contraction would then be a tetanic response on the part of some muscle fibres, while the desynchronized contraction would approximate more nearly to a twitch.

In some experiments desynchronization was also produced by applying the two stimuli to different parts of the nerve, and adjusting their time interval so that, as before, those fibres which were excited by the first

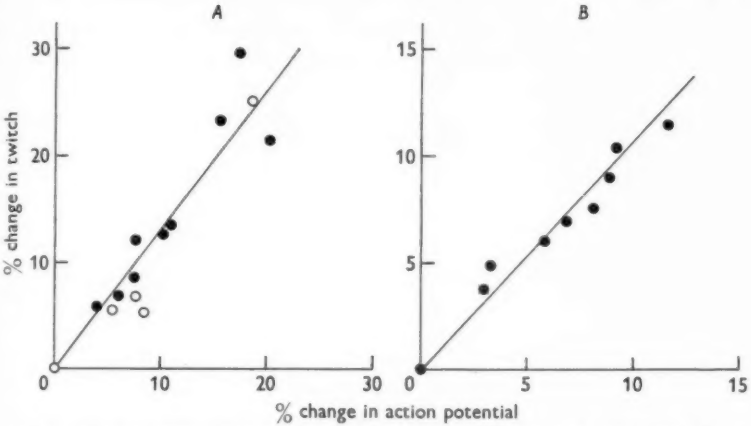

Fig. 4. The correlation between the change in the twitch and the change in the back-response on desynchronizing the nerve volley. *A*, results obtained from the whole series of experiments, each point representing one experiment (● gastrocnemius; ○ soleus). *B*, results obtained in a single experiment on gastrocnemius by altering the time interval between the two stimuli used to produce a desynchronized nerve volley. Ordinate: the difference in the peak tensions developed by the synchronized and desynchronized volleys, expressed as a percentage of the tension developed by the synchronized volley. Abscissa: the difference in the 'areas' of the back-responses developed by the synchronized and desynchronized volleys, expressed as a percentage of the area of the direct response plus that of the back-response developed by the synchronized volley. *B* is plotted on twice the scale of *A*. (The area of the direct response was the same for the synchronized and desynchronized volleys. The action potentials were recorded monophasically and their areas were measured by projecting an enlarged image of the record on squared paper.)

stimulus (submaximal) were refractory to the second stimulus (maximal). (The synchronized volley was usually produced by altering the interval of time between the two stimuli so that the submaximal shock then failed to excite the nerve.) In studying the medial head of gastrocnemius both stimuli were applied to the muscle nerve, for in this case it was easy to dissect enough of the nerve free to apply two well separated pairs of stimulating electrodes to it. In studying soleus the first stimulus was applied to a portion of ventral root other than that which was being recorded from

(if necessary the root was subdivided to obtain two portions). For both muscles it was thereby confirmed that desynchronization reduces the magnitude of both the twitch and of the back-response. Stimulation at two places ensured that the first stimulus was not producing any purely localized changes which might affect the response to the second stimulus. Merton (1954), using this method in man, where very long lengths of nerve may be studied, was able to separate the two half-volleys by as much as 10 msec. In the cat such wide separation could not be obtained and we have not investigated volley intervals of over 1 msec.

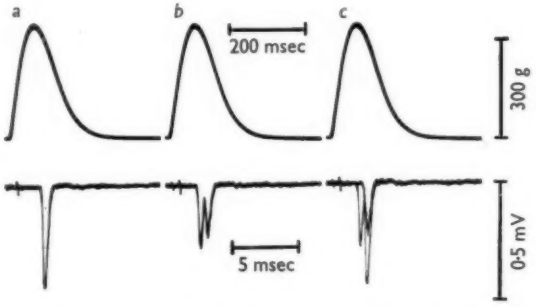

Fig. 5. The absence of effect on the twitch of desynchronizing the nerve volley in a preparation in which the back-response was naturally absent. Records of the contraction of soleus (above), and the action potential of its motor fibres (below). *a*, synchronized volley; *b*, desynchronized volley; *c*, superimposed responses to synchronized and to desynchronized volleys. (The compound action potential due to stimulation of the small motor fibres could just be seen in these records, but no back-response could be detected. Spikes retouched.)

Quantitative aspects. On desynchronizing the nerve volley in different preparations there has been a definite correlation between the change in the back-response and the change in the twitch. Figure 4*a*, derived from the whole series of experiments, shows the percentage change in the twitch plotted against the change in the back-response, expressed as a percentage of the total area of the compound action potential. There is an approximately linear relation, though no particular significance need be attributed to the precise slope of the line. The records chosen for measurement were in every case taken from the beginning of the experiment. Records taken at the end of the experiment or after fatiguing the muscle sometimes showed larger changes in tension relative to the back-response. It is possible that in these cases there was some degree of neuromuscular block and that the back-response initiated propagated muscle action potentials in some muscle fibres which were not excited by the direct response. (Lloyd, 1942, showed that the back-response

could be set up in nerve fibres the muscle fibres of which had not been activated.) In individual experiments a quantitative relation between the back-response and the twitch could be demonstrated by progressively increasing the time interval between the two nerve volleys, when the back-response and the twitch became smaller together. An example is shown in Fig. 4*b*, where the results are plotted in the same way as those of Fig. 4*a*.

Under certain conditions a back-response was not set up even by a synchronous nerve volley, and it is of great interest that no change could then be found in the twitch on desynchronizing the nerve volley. In four experiments out of eight on soleus no back-response was found at all; an example of this is shown in Fig. 5. In ten experiments on gastrocnemius the back-response was never naturally absent. It could, however, be temporarily abolished by fatiguing the muscle. This was achieved by

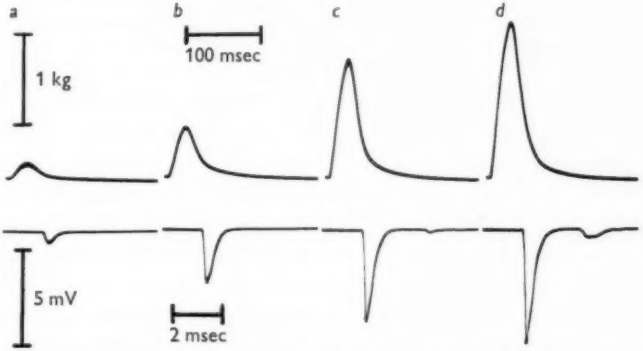

Fig. 6. The effect of the back-response of altering the strength of the stimulus so as to excite varying numbers of the large motor fibres. *a*, *b*, *c*, *d*, increasing stimulus strength (*d*, maximal). Above, contraction of the medial head of gastrocnemius; below, the action potential of its motor nerve fibres. (Spikes retouched.)

stimulating the muscle repetitively for 10–30 sec, while its blood supply was temporarily occluded by applying a clip to the popliteal artery. In confirmation of Merton (1954) it was found that the effect of desynchronizing the twitch could thereby be abolished, but in addition it was found that this only occurred when the back-response was also abolished. In addition in three preparations it was found that an injection of a large dose of anaesthetic abolished both the back-response and the effect on the twitch of desynchronizing the nerve volley.

Submaximal stimulation. Submaximal twitches were shown by Merton (1954) to have the time course of the desynchronized twitch rather than that of the synchronized twitch. In the present experiments it was found that the back-response was usually only produced by stimuli which excited

an appreciable fraction of the α motor fibres, as illustrated in Fig. 6. The absence of the back-response following the weak stimuli is most simply interpreted as due to the failure of the summed effect of the action potentials of the different muscle fibres to reach the threshold of the nerve fibres (cf. Lloyd, 1942, Figs. 2, 6). Thus, in agreement with the results already described, the two submaximal action potentials of a desynchronized nerve volley would be expected to produce a smaller back-response than does a synchronized nerve volley.

Repetitive stimulation. If the alteration in the back-response is accepted as the cause of the twitch desynchronization effect then no difference would be expected between the tetanic contractions produced by repetitive synchronized and by repetitive desynchronized nerve volleys; for repetitive discharge of the nerve fibres in response to each single stimulus could not then increase the size of the contraction. No difference was found in man between such contractions (Merton, 1954) and none has been found in the present experiments on the cat.

The effect of nerve stimulation during the muscle refractory period

During the course of the experiments a completely different method was discovered for reducing the back-response. This consisted in setting up a second action potential in the nerve immediately after the initial maximal synchronous volley. Figure 7 shows the abolition of the back-response by this means. It will be seen that the double stimulation also caused the contraction to become smaller, and as before this may be attributed to the reduction in the back-response. The nerve action potential set up by the second stimulus was apparently too early to re-excite the muscle. This was probably because the muscle was still refractory, and Eccles & O'Connor (1939) have shown that under similar circumstances an end-plate potential may be set up in the muscle without leading to a propagated muscle action potential.

Figure 8 shows that the somewhat paradoxical reduction of the contraction produced by the second stimulus only occurred for stimulus intervals above 0·6 msec and below 1·2 msec. For shorter intervals no second nerve action potential was excited, and for longer intervals the muscle was presumably excited by both action potentials. The second action potential only reduced the contraction in preparations in which a back-response was found after a single maximal shock. Thus these alterations in the size of the contraction may be explained by supposing that a second nerve volley may reach the muscle just too early to re-excite it, while the 'normal' back-response occurred just late enough to excite the muscle; and no other explanation is immediately apparent. The abolition of the back response by a second stimulus after an intermediate interval

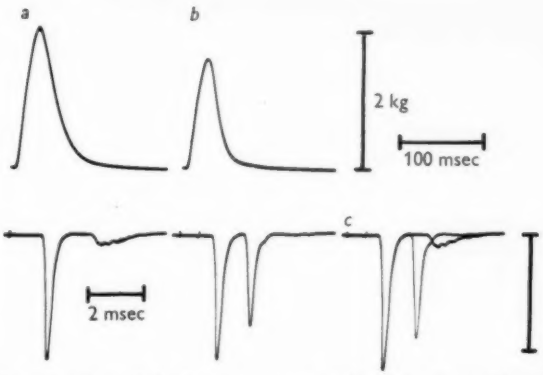

Fig. 7. The reduction of the back-response produced by re-exciting the nerve during the refractory period of the muscle. Above, contraction of the medial head of gastrocnemius; below, the action potential of its nerve fibres. *a*, single maximal shock; *b*, two maximal shocks separated by 0·7 msec; *c*, superimposed records of the action potentials set up by single and by double stimulation. The nerve action potential initiated by the second stimulus was too early to re-excite the muscle, but, by reducing the back-response, it made the muscle contraction smaller. (Earlier in experiment of Figs. 2 and 3. Spikes retouched. Voltage calibration, 3 mV.)

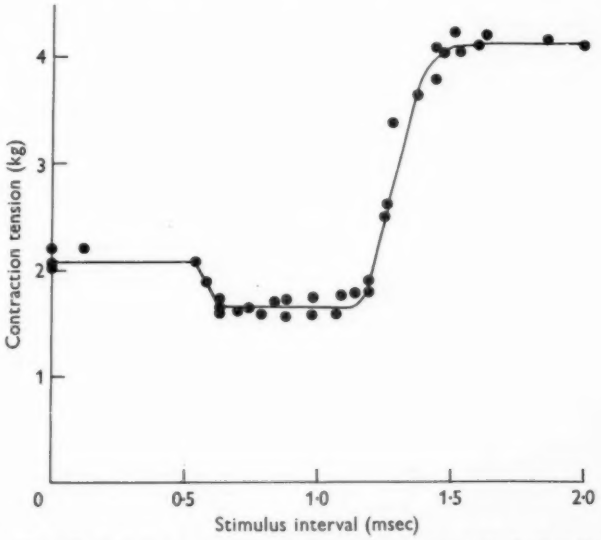

Fig. 8. The effect, in a preparation with a back-response, of altering the time interval between two maximal stimuli applied to the nerve. When the second stimulus excited a second nerve action potential, but the muscle was still refractory, the contraction was reduced, because the back-response was then reduced. (Same experiment as Fig. 7. Both stimuli were about twice the strength of that required to excite all the large motor fibres when they were not refractory.)

may be attributed to the refractoriness of the nerve fibres which was induced by the second action potential, and which prevented their re-excitation by the muscle action potential. Alternatively, it is possible that the second action potential collided with the back-response in the fine branches of the motor nerve, and that this prevented the back-response spreading into other branches of any axon in which it was excited. J. C. Eccles and W. J. O'Connor (unpublished observations) also sometimes observed such a diminution of the contraction with double stimulation, and likewise attributed it to a reduction of repetitive firing of the muscle (personal communication, J. C. Eccles).

Electrical recording of double discharges in muscle

By recording the electrical activity of the muscle it has been possible to confirm that a single shock to the nerve may excite a double discharge

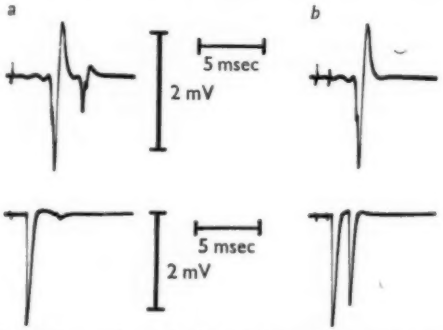

Fig. 9. A double discharge found in the electromyogram as a result of a single stimulus to the nerve. Above, the electromyogram recorded with fine electrodes placed on the surface of the muscle; below, the action potential of the motor fibres recorded from the ventral root. *a*, a single stimulus produced two waves of activity in the electromyogram, and a small back-response in the nerve fibres; *b*, stimulation of the nerve with two maximal shocks, the second falling within the refractory period of the muscle, abolished the second wave in the electromyogram, because the back-response was thereby abolished. (The contraction produced by the two stimuli was smaller than that produced by the single stimulus.)

in some muscle fibres. For this purpose the response of a localized portion of the muscle was recorded by applying to its surface a pair of fine wires, the tips of which were separated by about 0·1 mm (cf. Torrance & Whitteridge, 1948; Liddell & Phillips, 1952). These electrodes were spring-mounted so that they were free to move with the muscle as it contracted and they recorded potentials of approximately constant form in spite of successive contractions of the muscle. The action potential so recorded appeared to be derived from several motor units at least, for it did not

behave in an 'all-or-none' manner when the strength of the stimulus was varied. Figure 9*a* shows an electromyogram recorded when the nerve was excited with a single maximal stimulus. It will be seen that there were two separate waves of electrical activity, separated by about 2·5 msec at their beginning. When the nerve was stimulated with two maximal shocks, the second so timed that it fell within the refractory period of the muscle, then the second component of the electromyogram disappeared while the first remained unchanged (Fig. 9*b*). As again shown in Fig. 9, this arrangement of stimulation abolishes the back-response, and it may therefore be concluded that the second wave of activity in the electromyogram was due to the excitation of some of the muscle fibres by the back-response. Thus the two waves in the electromyogram represented the excitation of the muscle first by the direct response and then by the back-response. Such records were readily obtained in three preparations and demonstrated directly that some muscle fibres fire twice after a single shock is applied to the nerve.

Records such as those shown in Fig. 9 were not obtained from all sites on the belly of the muscle, and in many cases the amplitude of the second phase of the electromyogram was considerably smaller. Such double firing has, however, been observed in three preparations from over fifty recording sites on either soleus (two experiments) or gastrocnemius (three experiments); (in one of these three experiments the back-response was not recorded directly, but its presence was deduced from the change produced in the twitch by applying a second stimulus during the refractory period of the muscle). It is perhaps of interest to note that in initial experiments with belly–tendon leads the potentials recorded could not be analysed with a certainty into two separate components, because they were of much longer duration than those later recorded with the fine electrodes (cf. Eccles & O'Connor, 1939). Even with the fine electrodes the action potential sometimes lasted for several milliseconds, and the separate waves due to double firing were not then conspicuous. In some such cases a polyphasic record was obtained, the individual spikes of which were not unlike those due to repetitive firing. These, however, did not alter when the nerve was re-excited during the refractory period of the muscle, and were probably due to the existence of separate groups of muscle fibres, the end-plates of which lay at different distances from the recording electrodes.

When the strength of a single stimulus to the nerve was varied the second spike of a double discharge only appeared when the stimulus was large enough to excite a back-response (cf. Fig. 6). Reduction of the second phase of the electromyogram could also be produced by desynchronizing the nerve volley, thereby reducing the back-response (cf. Fig. 3). In this case, however, the form of the first wave of electromyogram was also altered because of the change in synchronization of the potentials of different motor units. The effect on the electromyograms of desynchronizing the nerve volley does not, therefore, provide such unequivocal evidence in favour of double firing as does the reduction of the back-response by a second maximal nerve volley falling in the refractory period of the muscle, for this does not alter the form of the first wave of the electromyogram. Indeed, it is this type of experiment which proved that the two waves in the electromyogram were usually due to double excitation of the muscle, rather than to the existence of two groups of muscle fibres the potentials of which reached the electrodes at two different times.

DISCUSSION

The present experiments have shown that in the anaesthetized cat the effect on the twitch of desynchronizing the motor nerve volley can be adequately accounted for in terms of alterations in the back-response of the motor nerve fibres. This explanation appears sufficient also for the effects found by Merton (1954) in the unanaesthetized man. Certainly it appears unnecessary to postulate any mechanism for the twitch effect which depends upon assuming properties of muscle which have not been established by other means.

It is, of course, the synchronized twitch rather than the desynchronized twitch which is abnormal, for in the synchronized twitch some muscle fibres appear to be re-excited by the back-response and to be contracting tetanically. It is remarkable that the back-response is excited late enough to have such an effect, and if it were only slightly earlier it would fail to re-excite the muscle fibres because they would still be refractory. The latency in the development of the back-response might occur for either of two reasons. Most probably it occurs because the stimulus of the muscle action potential is not effective until the nerve fibres have largely recovered from their relative refractory period. On the other hand, the back-response may be excited in the fine terminals of any motor axon, and spread only slowly from there to the other branches of the same axon. At any rate the effect appears to be common in the two muscles studied, and as these were chosen solely for their experimental convenience it is probably common in other muscles as well.

Eccles & O'Connor (1938, 1939) have previously described repetitive electrical discharges of normal muscle following single stimuli to the nerve. These discharges sometimes persisted for up to 100 msec, and it is conceivable that they were due to excitation circling from nerve to muscle and back again, rather than to changes restricted to the motor end-plate. Eccles & O'Connor found large effects only in decerebrate animals, and these may have been partly due to exposing the muscles in a moist chamber, which might be expected to increase the excitability both of the muscles and their nerves by lowering their carbon dioxide content. After the injection of eserine, prolonged repetitive discharges may be found in the nerve (Masland & Wighton, 1940; Feng & Li, 1941) but this is not the sole cause of the repetitive discharge of the muscle (Eccles, Katz & Kuffler, 1942.)

The effect of a synchronous nerve volley in doubly exciting a muscle appears unlikely to be of any importance for the physiological control of muscle, for this normally depends upon dicharges which are asynchronous in different nerve fibres. In addition, the degree of synchronization

required is so great that even a tendon jerk is unlikely to be sufficiently synchronized to evoke a back-response (cf. Fulton & Liddell, 1925). The back-response is therefore probably a 'physiological artifact', and its existence emphasizes the dangers of investigating the function of the nervous system by initiating synchronous volleys of nerve impulses.

SUMMARY

1. In anaesthetized cats the compound action potential set up in the motor fibres on stimulating an intact muscle nerve (soleus or medial gastrocnemius) was recorded from a ventral root. The direct response of the α motor fibres to a single stimulus was followed by a 'back-response' due to restimulation of the same nerve fibres by the muscle action potential. Comparison of the time relations of the action potentials with the refractory period of the muscle suggested that the back-response would re-excite the muscle fibres.

2. When, by means of double stimulation, the nerve volley was desynchronized into two half-volleys separated by about 0·6 msec, the back-response was greatly reduced. Simultaneous recording of the muscle contraction showed that the twitch then became smaller and shorter, as described by Merton (1954).

3. In the whole range of experiments the magnitude of the change of the twitch on desynchronizing the nerve volley could be correlated with the magnitude of the simultaneous change in the back-response. A similar result was obtained in individual preparations by altering the time interval between the two nerve volleys.

4. In four preparations the back-response was absent, and in these the effect on the twitch of desynchronizing the nerve volley was also absent. In some other preparations both effects were abolished together by fatiguing the muscle, or by increasing the depth of anaesthesia.

5. The back-response set up by a single maximal stimulus could also be reduced by setting up a second action potential in the nerve immediately after the first. This again reduced the size of the muscle contraction, for the second nerve action potential was too early to re-excite the muscle, the refractoriness of which outlasted that of the nerve.

6. The electromyogram, recorded from the surface of the muscle with fine electrodes, might show two waves of electrical activity following a single stimulus to the nerve. The latter wave was reduced when the back-response was made smaller, and appeared to be due to double firing of some muscle fibres.

7. It is concluded that in mammalian muscle the 'twitch' elicited by a single maximal stimulus applied to a muscle nerve may include the

response of some muscle fibres contracting tetanically, as a result of their restimulation by the back-response. The effect on the twitch of desynchronizing the nerve volley is then explained by the concurrent change in the back-response.

We should like to thank Mr E. T. Giles for technical assistance.

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THE LOCATION OF ADENINE NUCLEOTIDE IN FROG'S STRIATED MUSCLE

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In an earlier paper (Hill, 1959*a*) the location of tritium-labelled adenine nucleotide was studied by autoradiography in a freeze-dried striated muscle of the frog. The results were considered to be inconclusive, mainly on the grounds that the imperfectly preserved structure provided by freeze-drying might well be associated with a disorganized distribution of the nucleotide. In addition, the danger of disturbance by water, even in the 'water-proofed' sections, was apparent. Similar experiments have therefore been done with chemical fixative, incorporating a nucleotide precipitant, instead of freeze-drying. The improvement in fixation gives perfect visible structure, but by sacrificing the very rapid freezing in favour of slow-acting chemicals penetrating from the outside, one uncertainty has been exchanged for another; for there is every possibility that mobile substances may change their siting during chemical fixation. Indeed, as will be seen, gross movement of the labelled nucleotide does in fact take place. The artificial distribution so created is not, however, necessarily irrelevant to a study of normal muscle; there are grounds for arguing that the distribution which is found, when considered in relation to the striation boundaries, that is to say the *longitudinal* as distinct from the *transverse* distribution, is the same as in the living state.

A further objection to the earlier technique was in connexion with its reliance on an isolated muscle remaining in normal conditions during a prolonged period of soaking in a strongly radioactive mixture, this being necessary to label the muscle nucleotide from an external solution of radioactive adenine. The alternative, namely, injection of a living frog followed after a suitable time by dissection of the labelled muscle, should ensure normality immediately before fixation. Injection involves the use of larger quantities of radioactive material, several millicuries being needed. With carbon-14 it was impracticable to achieve sufficient activity for autoradiography by injection, so the method of using an isolated muscle was therefore developed. With tritiated adenine at commercial prices injection would likewise involve prohibitive amounts (costing several hundred

pounds for each experiment), but the 'home-made' product, prepared in the way described by Hill (1959*a*) can be used in the quantities required in a limited number of experiments. The tritiated adenine was therefore administered by injection in the experiments to be described.

The birefringence of the muscle was made use of in the earlier experiments for viewing the striations. It has now been found possible to stain the muscle through the autoradiographic film; this has led to a marked improvement in accuracy in relating the silver grains with the various regions of the sarcomere. The stain pattern within the sarcomere, though there is uncertainty as to the identity of the stainable material, has incidentally proved interesting.

This paper is mainly concerned with resting muscle. In addition an attempt has been made to find out what happens as the result of activity, and two experiments have been done in which the muscles were fixed in a state of contracture and exhaustion following poisoning. Although the disorganization of the structure resulting from such severe treatment has made it impossible to investigate the isotope location in relation to the boundaries of the A and I bands, certain conclusions regarding the state of mobility of the nucleotide in activated muscle have been derived.

A glycerol–water-extracted muscle has also been examined.

METHODS

Labelling the muscle. To label the muscle at a level of radioactivity comparable with that found suitable in the earlier experiments, i.e. at not less than 50 μc/g, it was calculated (from data given by Hill, 1959*a*) that a frog must receive an injection of about 200 μc/g, amounting to a total of 4 mc in a 20 g frog. In mice (Brues, Stroud & Rietz, 1952) the 30-day median lethal dose is about 1 mc/g body weight, so the amount proposed was not likely to be harmful.

The tritiated adenine was of the same batch as that used earlier (Hill, 1959*a*). After 20 months storage *in vacuo* it was assayed spectrophotometrically and was found to have undergone no break-down. It was therefore not considered necessary to assay it again for radioactivity, and the earlier assay was simply corrected for natural decay.

Two frogs were injected with a solution of the tritiated adenine in water, the injection being made intraperitoneally in the lower abdomen to the left of the mid line.

Frog 1. Weight 20 g. 4·6 mc ^{3}H-adenine injected in 2·0 ml. water (230 μc/g).

Frog 2. Weight 19 g. 3·18 mc in 1·75 ml. water (167 μc/g).

The frogs were then kept in a moist atmosphere at 15° C for 47 hr (frog 1), and 24 hr (frog 2). Bennett & Krueckel (1955*b*) showed that, in mice, adenine is converted to nucleotide within 2 hr of injection, and it was thought that 24–48 hr, in the frog, would be sufficient for the conversion. (These times may, in fact, have been too long, for there is some indication that excretion had started.)

Both sartorius muscles were then dissected from each frog. After soaking for 1 hr in Ringer's solution (mM: NaCl 96, KCl 5·0, $CaCl_2$ 4·0) the four muscles were treated as follows:

Muscle 1 (frog 1). Fixed in a normal resting condition, following pre-treatment (see below) designed to minimize the 'stimulating' effect of the osmium fixative.

Muscle 2 (frog 1). Fixed at the peak of a caffeine contracture.

Muscle 3 (frog 2). Fixed after extraction by glycerol–water.

Muscle 4 (frog 2). Fixed after poisoning with cyanide and iodoacetate, followed by stimulation to exhaustion.

The pre-fixing procedures. An excitable muscle is stimulated, and shortens as the result of immersion in an osmium tetroxide fixative. If the muscle is first made inexcitable by soaking in isotonic potassium sulphate it is no longer affected by the fixative. The fixative therefore seems to produce an effect on the muscle similar to that of the depolarizing action of potassium. It has been stated (Frank, 1958) that withdrawal of calcium from the extracellular fluid completely prevents the mechanical response of a muscle when potassium is applied. Experiments similar to Frank's were done but it appears that the response is actually never entirely abolished. A thin muscle, the m. ext. long. dig. IV as used by Frank (1958), was soaked in the following solution: isotonic choline chloride; 5 mM TRIS (tris-(hydroxymethyl)-amino methane) buffer, pH 7·4; 2 mM K_2SO_4; 10^{-4} (w/v) tubocurarine chloride. The response to isotonic K_2SO_4 was tested after varying times of exposure. The mechanical response was found to be greatly diminished, both in peak and duration, but it was never entirely absent. The shortening caused by the osmium fixative also was not completely prevented by such withdrawal of calcium. Deprivation of calcium seems to have an effect rather similar to that described by Denton (1948) where no total abolition of response was found.

A similar reduction in response to depolarization is found with the sartorius. At 0° C a normal sartorius attached to an isotonic lever shortened 100 units when immersed in isotonic K_2SO_4. After soaking for 60 min in the above mixture the response was reduced to 17 units, but within 20 min of returning the muscle to normal Ringer's solution the response had returned to 77 units.

Although withdrawal of calcium does not eliminate the response to depolarization or to immersion in the fixative, it was considered to be a useful pre-treatment of the muscle with the object of reducing the disturbance of equilibrium as far as possible. In addition, it was thought to be desirable to allow the muscle to recover from such stimulus as still remained. It seemed better, therefore, that the stimulus should result from immersion in K_2SO_4, followed by a suitable recovery period, rather than as the result of contact with the fixative, after which recovery might be inhibited.

Muscle 1 was soaked in oxygenated, Ca-free mixture (of above composition) at 0° C for 48 min. It was then fully stretched on a glass support and immersed in isotonic K_2SO_4 for 15 min at 18° C. It was then fixed.

Muscle 2 was stretched to nearly full extension on a glass support and immersed in oxygenated Ringer's solution, containing 0·2 % caffeine, at 18° C. In a previous trial the time course of the ensuing contracture had been established, and at the estimated peak of contracture, 5 min after immersion in the caffeine, the muscle was fixed.

Muscle 3 was made inexcitable in isotonic K_2SO_4 and was then soaked for 65 min in 50 % (v/v) glycerol–water buffered at pH 7·4 with 5 mM TRIS; it was then fixed.

Muscle 4 was connected to an isotonic lever and immersed in Ringer's solution buffered at pH 7·4 with 5 mM TRIS, containing 0·4 mM iodoacetic acid and 1·0 mM sodium cyanide. The temperature was kept at 0° C for 60 min to allow penetration of the poison, and was then raised to 19° C. The muscle was stimulated with single shocks at 1/sec, with the tension on the muscle raised to 18 g to prevent contracture. After 200 shocks the twitch response had vanished and the muscle was fixed.

Fixative. Buffered osmium tetroxide was used as the protein fixative, with lanthanum nitrate added to precipitate the nucleotide as the insoluble lanthanum salt. The mixture had the following composition (mM): osmium tetroxide 40 (1 %); TRIS 39; HNO_3 31 (pH 7·4); $La(NO)_3$ 28; sucrose 110. The sucrose was added to increase the osmotic pressure of an earlier mixture (isotonic with Ringer's solution) in which the myofibrils had become separated by what was thought to be osmotic swelling of the muscle during fixation. The

total osmotic pressure of the above mixture is 50 % greater than that of Ringer's solution. In this the muscle obviously undergoes some shrinkage; it might be better to add a smaller amount of sucrose. The muscles, after the pre-treatment described and still mounted on their glass supports, were plunged into the fixative at room temperature (18° C) and left for 2–3 hr.

The fixative materials had then to be removed, and water replaced by ethanol, without allowing the precipitated lanthanum nucleotide to dissolve. It was therefore necessary to avoid lanthanum-free aqueous or partially aqueous solutions. The muscles were first soaked for about 3 hr in an aqueous solution of 10 mM lanthanum nitrate buffered with 110 mM collidine (2,4,6-trimethylpyridine) adjusted to pH 7·2 with nitric acid. The collidine was used, at this stage, in preference to TRIS, because the former is soluble in ethanol. The muscles were then transferred to ethanol containing 1 % by volume of the same lanthanum–collidine mixture, and put into an oven at 37° C during the night to expedite the exchange of water and ethanol. The muscles were then soaked for some hours in pure ethanol to remove the last traces of water, lanthanum and collidine (lanthanum nitrate is soluble in ethanol).

The muscles were then cut up into thin strands for longitudinal sectioning, or into thin transverse slices, or into slices cut at about 45° to the long axis. These pieces were left for a further period of about 1 hr in ethanol. The ethanol was then replaced by methacrylate monomer (5 % methyl: 95 % *n*-butyl) and embedded in No. 00 capsules by polymerization at 65° C with 2 % 2,4-dichlorobenzoyl peroxide as the activator.

Sectioning and autoradiography. The sections, 0·2–1·0 μ, were cut on a Cambridge rocking microtome with a glass knife. The first autoradiographic tests with this material showed that the low solubility of the precipitated nucleotide, combined with the low permeability of the methacrylate were together sufficient to prevent any loss of activity when the sections were allowed to come into contact with water: there was no need for any of the elaborate measures required in the earlier experiments to prevent loss of nucleotide. The sections were therefore floated off the microtome knife, transferred to warm water (40° C) to allow flattening, and picked up on slides prepared with gelatine adhesive (Hill, 1959*a*) and dried. They were mounted with autoradiographic stripping film (Kodak A.R. 10) in the usual way. After the time allowed for exposure the autoradiographs were developed in Kodak D. 76 fine-grain developer, fixed and washed.

Staining the muscle sections. It was found possible to stain the sections with basic dyes, without at the same time producing a dense coloration of the over-lying autoradiographic film, by the use of stain solution buffered at pH 4. The pK of protein carboxyl groups is around 5, and at pH levels below this the carboxyl binding of most basic dyes is negligible. At pH 4 a tissue section will take up stain, and it seems likely (Swift, 1955) that this is due to combination of dye with the phosphoryl groups of nucleic acid and of nucleotide. With basic dyes the combining group is the amino group which exists in a positively charged dissociated form ($—NH_3^+$) except at high pH levels. The primary phosphoryl groups of nucleic acid have a pK of about 2, and are therefore negatively charged at pH greater than 2. Dye-nucleate, and probably also dye-nucleotide complexes are formed through salt linkages between cation and anion, and the dye cation will compete with other cations (e.g. La^{3+}) for binding sites. Provided, therefore, the pH lies between 2 and 5 the dye should be able to stain the nucleotide of the muscle but not the protein either of the muscle or of the autoradiographic film. This differential method of staining was tried and found to work well.

Free nucleotide is not stained. The reason for this is not clear, for the pK value of at least one of the phosphoryl groups of ATP is below 4, but it seems probable that since combination of the dye with free nucleotide does not result in the formation of an insoluble compound, such free nucleotide as is able to combine with the dye in competition with lanthanum is lost into the solution.

Procedure for staining. The washed slide, comprising section and autoradiograph, was immersed for 15–60 min in the stain solution, consisting of 20 mM crystal violet in 100 mM pH 4 acetate buffer. The uncombined dye was then washed out by immersing the slide in several changes of 10 mM pH 4 acetate buffer. After 20–30 min the gelatine of the autoradiographic film was found to be practically colourless, with the muscle section remaining darkly stained. The slide was then washed in distilled water, and dried. Euparal was used as the mountant. This prolonged procedure never resulted in loss of contact between the emulsion and the muscle tissue.

The autoradiographs were examined with a high-power oil-immersion objective (N.A. 1·3), and photographed on Kodak P. 200 plates, with a colour filter to provide the required contrast. The relationship between the stained regions and the A and I bands was established, whenever necessary, by using polarized light.

RESULTS

Preliminary experiments with ^{14}C-adenine

In the earlier experiments (Hill, 1959*a*) it was necessary to establish the chemical identity of the bound radioactive material; this was shown chromatographically to be adenine nucleotide. With the new method of labelling the muscle by injecting the frog it did not seem necessary again to go to the length of chromatographic separation of the radioactive constituents, provided the muscle from an injected frog stood up to certain simple tests with ^{14}C-labelled adenine. With this isotope it is possible to make whole-muscle measurement of radioactivity by using a Geiger–Müller counter (Hill, 1959*a*).

A 20 g frog was injected intraperitoneally with 77 μc ^{14}C-adenine (Batch 9, 9·7 mc/m-mole, obtained from the Radiochemical Centre, Amersham, Bucks.). After 24 hr both sartorii were dissected, and put into oxygenated Ringer's solution. The emission from the surface of the muscle was measured in the way described previously (Hill, 1959*a*). The counting rates (counts/min) were as follows, with the times (min) after dissection given in brackets:

Muscle 1. 536 (49); 530 (52); 496 (56); 465 (59); 460 (74); 523 (77); 517 (80); 516 (84); mean value 509 counts/min, equivalent to 1·41 μc/g (conversion factor given by Hill, 1959*a*);

Muscle 2. 515 (62); 500 (64); 447 (68); 459 (71); 478 (86); 475 (89); 488 (93); 500 (96); mean value 483 counts/min, equivalent to 1·34 μc/g.

It is seen that there is practically no decline from the first measurement on each muscle. The radioactivity is therefore bound within the fibre in the living state, and is not due to unchanged adenine. The levels, for the two muscles, of 1·41 and 1·34 μc/g muscle, for an injection of 3·8 μc/g frog, are consistent with values obtained before (Hill, 1959*a*).

The second point to be tested concerns the proportion of bound to unbound activity contained within the fibre. When there is equilibrium

between the activities of the various forms of nucleotide about 90% of the activity should be lost when the muscle is killed by immersion in glycerol–water, or pure water.

To test this, one muscle was immersed in 50% glycerol (v/v), buffered with 5 mM TRIS at pH 7·4. The ensuing counting rates (per minute) were as follows, with the times (min) after immersion given in brackets:

Muscle 1. 132 (15); 93 (27); 77 (52); 89 (68); 61 (95); 61 (100); 49 (104); 50 (110).

The activity bound against release by water, but freed by acid, was then measured by immersing the muscle in ice-cold 10% trichloroacetic acid in 50% glycerol–water:

54 (12); 101 (17); 90 (22); 88 (27); 88 (32).

The second muscle was immersed in pure water. The counting rates (per minute) were as follows, with the times (min) in brackets:

Muscle 2. 244 (17); 95 (54); 101 (71); 86 (97); 86 (102); 92 (108).

The muscle was then put into ice-cold 10% trichloroacetic acid:

56 (14); 60 (19); 64 (24); 51 (29); 51 (34).

The interpretation of these figures is complicated by swelling and shrinkage of the muscle as the result of immersion in the different solutions. Absorption of most of the radiation within the muscle means that a swollen muscle will appear to have less, and a shrunken muscle more radioactivity than it would have done at normal volume. Water causes swelling, glycerol and trichloroacetic acid give shrinkage. The final weights of the muscles were: muscle 1, 52 mg; muscle 2, 73 mg. The final widths were practically the same, so the ratio of thicknesses was 52/73, which is approximately the reciprocal of the ratio of the final counting rates; in other words the total contents of activity are roughly the same in the two muscles, as they should be.

The living muscle was not weighed, but it was obvious that the volume of the acid-treated water-extracted muscle was rather less than that of the living muscle, and it follows that the final counting rate at normal volume would be less than 50 counts/min. Thus the final content of activity is rather less than 10% of the normal value. This is appreciably higher than the residual value in the muscle rendered radioactive by the earlier method; a value of less than 3% was then found (Hill, 1959*a*). Further, the activity after water extraction, but before immersion in acid, was rather more than 19% of the original value, compared with a figure of something over 5% in the earlier experiments.

These differences are substantial, but they are not taken to indicate any essential discrepancy with the results obtained in the previous experiments.

The explanation may lie in a difference between the specific activities of the free and of the bound fractions of the nucleotide. In mice the specific activity of the nucleic acid adenine 'lags' behind that of the acid-soluble nucleotide (Bennett & Krueckel, 1955*a*): during the incorporation phase the former is the lower, during excretion it is the higher. In mice excretion occurs quite rapidly, and it is not improbable that at the time of dissection the frog's muscles had already passed their peak contents; this would account for the relatively high value of the activity bound against water and acid extraction.

An unexpected point emerges from the figures for the activity of the glycerol–water-extracted muscle. It is clear, especially in view of the shrinkage caused by glycerol, that there is a much greater loss of activity in the buffered glycerol–water than there is in pure water, and it takes place more rapidly. The remainder after this first phase of extraction is less than 10% of the original value; it is less than half of the remainder following water extraction. When the muscle is then immersed in acid there is no further loss of activity (the *increase* in counting rate must be attributed to further shrinkage of the muscle). Thus the glycerol–water (pH 7·4) has caused the extraction of the fraction which is resistant to the action of pure water, but which (after water) only yields to the action of acid.

These conclusions, it should be remembered, apply only to the outermost surface layer of the muscle; carbon-14 radiation is reduced by one half in its passage through as little as 30 μ of water. The results with autoradiography show that the isotope concentration averaged for several areas in the interior of the glycerol–water-extracted muscle corresponds to the higher retention found after extraction by pure water, so the acid-like effect of glycerol–water may be confined, in the allotted time, to the extreme surface of the muscle. The situation in the glycerol-treated muscle is, to judge from the variable stain pattern described later, in a state of flux, and the discrepancy may well be due to this.

Autoradiography: normal muscle

Unstained transverse sections. An autoradiograph of a transverse section of the normal sartorius is shown in Pl. 1, fig. 1. This autoradiograph has been over-developed so that, in this low-power photograph, the distribution of the isotope may be seen from the degree of blackening of the emulsion; the individual grains are not discernible. It is seen that the activity is not uniformly spread throughout the fibres, but that high concentrations occur near the surface in many of them. The degree of non-uniformity is variable; near the surface of the muscle the activity is more evenly distributed than it is in fibres which are more deeply placed

within the muscle. High-power photographs of individual fibres are shown in Pl. 2, figs. 3, 4.

This aggregation of nucleotide in regions near the surfaces of the fibres must be attributed to the presence of the lanthanum nucleotide precipitant. The lanthanum renders insoluble any nucleotide which it encounters on penetrating the fibre surface and further nucleotide diffuses into the 'vacuum' so created. The extent of the non-uniformity depends on the rate of penetration of the lanthanum and on the transverse mobility of the nucleotide within the fibre. Proximity with the muscle surface means a more rapid uptake of lanthanum, and this accounts for the greater uniformity of distribution in fibres near the surface. Deeper in the muscle the lanthanum enters the fibres more slowly, and the nucleotide has time, so to speak, to diffuse to the surface to meet it and the precipitation goes to completion at one side of the fibre before the fibre as a whole is invaded by the lanthanum.

There is one feature about the distribution which is difficult to understand. Not a single fibre seen in Pl. 1, fig. 1, or elsewhere has more than one zone of high concentration. The lanthanum approaches the fibres from both surfaces of the muscle, and in a fibre near the middle the aggregates might be expected to occur more or less equally on the two sides. Actually the fibres near the centre show a greater, rather than a smaller degree of one-sidedness as compared with fibres nearer the surface. It seems that an unstable situation must arise at some stage to account for this, so that as soon as precipitation of nucleotide starts at one focus it proceeds to completion 'explosively' with that focus as centre, and the participation of any other focus is excluded. It is possible that such a situation might occur because of a sudden local increase in the permeability of the muscle membrane to lanthanum.

It is necessary to explain why the non-uniform distribution of nucleotide in transverse section is attributed to events occurring at the stage of fixation rather than to causes operating earlier, even before dissection. In the first place, the aggregation always occurs on one or other of the sides of the fibre which face towards the surface of the whole muscle. Before dissection the surface is, in effect, not present, for blood vessels would then be the centres from which exchange occurs and to which gradient lines in distribution would be likely to point: the directions of the gradients in the various fibres should, if the non-uniformity were of pre-dissection origin, be randomly disposed. Secondly, if aggregation had occurred after dissection, but before fixation, some reason has to be given to explain the preference for aggregation near the surface. None can be suggested. It might be thought that nucleotide becomes concentrated in regions with a high partial pressure of oxygen; but it will be seen later that in a muscle

poisoned with cyanide the same unevenness in distribution is found. If the fibres had become permeable to nucleotide they would lose activity from the surface first of all, leaving the centre richer, not poorer than the surface. In any case the evidence is against any suggestion that an appreciable amount of isotope has been lost. In fact, the only acceptable explanation is that the patches of high concentration are caused by precipitation of nucleotide at those sites, the build-up being produced by diffusion of further isotope into the 'vacuum' so created.

On looking at the distribution (Pl. 1, fig. 4) in the glycerol–water-extracted muscle it is seen that the specific activity of the nucleotide in the different regions of the cross-section is practically constant. It is not reasonable, therefore, to argue that the irregular distributions seen in the other muscles are due not to variable concentrations of nucleotide, but to a variation in the specific activity of the nucleotide at different points across the fibre. In any case such unevenness of specific activity would have had its origin before the muscles were dissected, and the lines of gradient would be found directed at the blood vessels, not at the muscle surface.

The concentration of isotope in the muscle. The uneven distribution of isotope in the fibre cross-section means that an over-all estimate of activity in the muscle has to be made by counting grains over the entire cross-section of one or more individual fibres. A $0{\cdot}4\,\mu$ cross-section, cut at 45° to the muscle axis, exposed for 5 days, gave a total grain yield of 920 over one selected fibre; this works out as 4·9 grains/$100\,\mu^2$.day. The isotope content of the tissue is calculated on the same basis as before (Hill 1959*a*): (1) Each electron yields one grain. (2) Self-absorption within a $0{\cdot}4\,\mu$ section accounts for a loss of 45% of the total emission. (3) One half of the electrons are emitted towards the emulsion. For this fibre the calculation gives 140 μc/g muscle. The frog (No. 1) was injected with 230 μc/g. The content of this particular fibre, deep in the muscle, is therefore higher by a factor of about 2·2 than that shown by the carbon-14 tests for fibres near the surface of the muscle. The latter also appear in Pl. 1, fig. 1, to have a somewhat lower isotope content than that of the deep fibres.

Stained longitudinal sections. An autoradiograph superimposed on a stained longitudinal section of the normal muscle is shown in Pl. 2, fig. 1. Comparison with a similar specimen produced by the earlier technique (Hill, 1959*a*, Pl. 2) shows the present improvement in clarity of grains and striations.

The grain counts in longitudinal sections were found to vary over a tenfold range, as is consistent with the distribution seen in transverse section. Counts were made from areas of low, moderate and high

concentration. The results were as follows, with the estimated isotope concentrations given in brackets:

High:	11·7 grains/100 μ^2.day (230 μc/g)
Moderate:	3·9 grains/100 μ^2.day (71 μc/g)
Low:	1·2 grains/100 μ^2.day (22 μc/g)

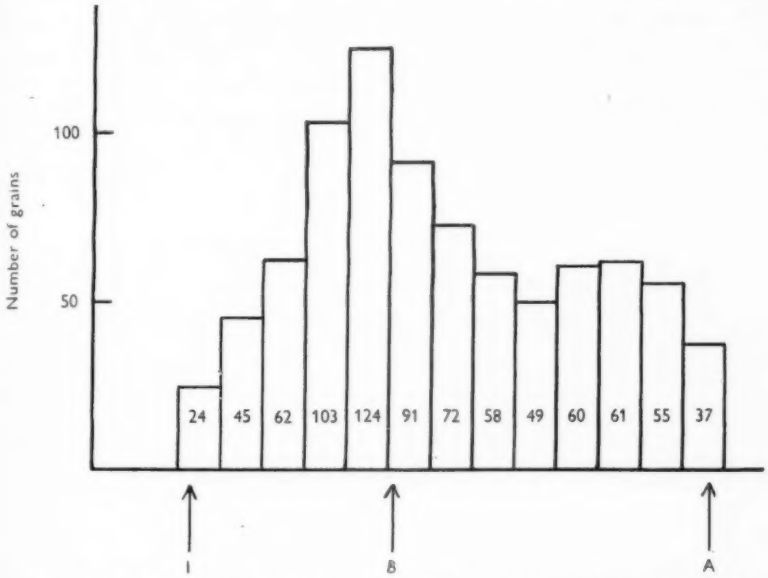
The sections were 0·8 or 1·0 μ thick, and self-absorption accounted for a loss of 60 or 66 % of the total emission.

These figures should be compared with the average value of 140 μc/g for the fibre as a whole. It is clear that the lowest concentration is probably no higher than that found in a specimen from which the free, extractable nucleotide had been removed; in other words, practically the whole of the mobile nucleotide has diffused out of the regions of lowest concentration to take the place of that precipitated elsewhere.

Analysis of distribution. Analyses of grain distribution in relation to the centres of the A and I bands were made for areas corresponding to the high, moderate and low grain yields. These analyses were done in the way described elsewhere (Hill, 1959*a*). The results are shown in Text-figs. 1, 2, 3. It is seen that the distributions are essentially the same irrespective of the grain density, showing that the bound nucleotide has the same distribution as has the fraction with transverse mobility. In each case there is a distinct peak close to the A–I boundary, on the I side of this boundary. In other words, there are two lines of aggregation in each I band, one on each side of the Z line. The distribution of grains towards the Z line falls off steeply: on the other side, towards the centre of A, the fall is less steep, and there is a slight indication of a further peak as the centre of A is approached. The over-all width of the I band is 1·0 μ and the centre of the peak in the distribution is only about 0·9 μ from that of the other member of the pair; yet it appears that these two lines of concentration have been clearly resolved apart. Reference to Hill (1959*a*, Text-fig. 7) shows that discrimination of this order should be possible with tritium, but the steepness of the descent from the peak is greater than would have been expected, even if the effective diameter of the grain profile which is presented to the emitted electrons is taken as 0·15 or 0·2 μ, rather than 0·3 μ as hitherto suggested. The assumptions made previously regarding the resolving power with tritium were based on very scant evidence, and it would seem that the actual power of discrimination is rather better than had been concluded from the previous calculations.

The total quantity of radioactive material which would have to be situated in the position of the peak to give the distribution which is actually found is estimated by adding up the total number of grains in the part of the distribution which lies on the I side of the peak. This implies an

absence of any other source in the I band. The sum is equal to one half of the total number due to the source assumed to be lying in the position of the peak. The remainder, all on the A side of the peak, have to be



Text-fig. 1. Distribution of grains over a section of normal muscle. Area with high nucleotide content (230 μc/g). A total of 841 grains is represented. These are grouped in 13 blocks between the centre of the I band (arrow at left) and the centre of A (arrow at right). The arrow at *B* is placed 0·75 μ from *A* and represents the position of the A–I boundary. Sarcomere length 2·5 μ. Muscle section 0·8 μ thick; exposure 11 days.

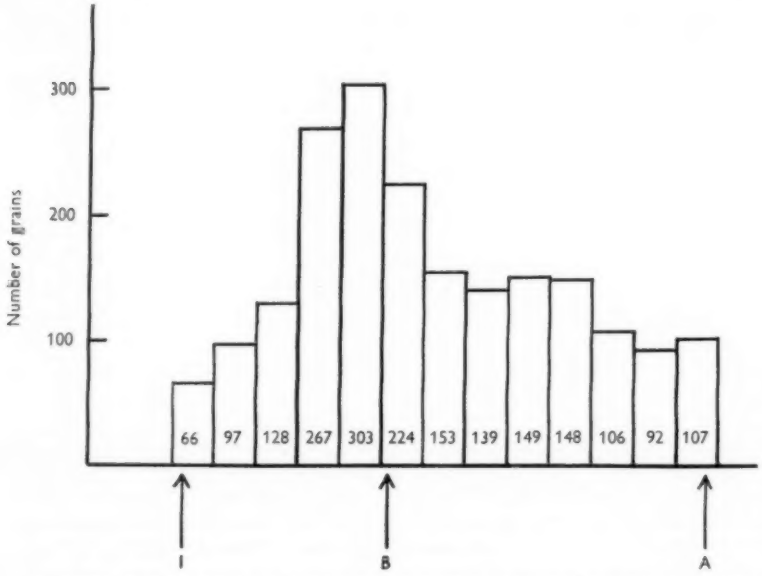
accounted for by some other source. The proportion lying in the I line-deposit is thus calculated. In Text-figs. 1, 2, 3 the number of grains attributable to the I line-deposit is twice the sum of the grains shown in blocks 1, 2, 3, 4, and half of 5. The results are as follows:

Region	Grains due to line-deposit in I	Total	Percentage aggregated in I lines
High density	592	841	70·4
Moderate density	1420	1979	71·7
Low density	536	801	66·9

Thus the distribution is such that it could be accounted for if about 70% of the total isotope present in the muscle were aggregated in narrow disks lying on either side of the Z line. The position of these disks is such

that, in accordance with the usual nomenclature, they may be called the N bands. It will be seen below that basophilic material lies in these same regions.

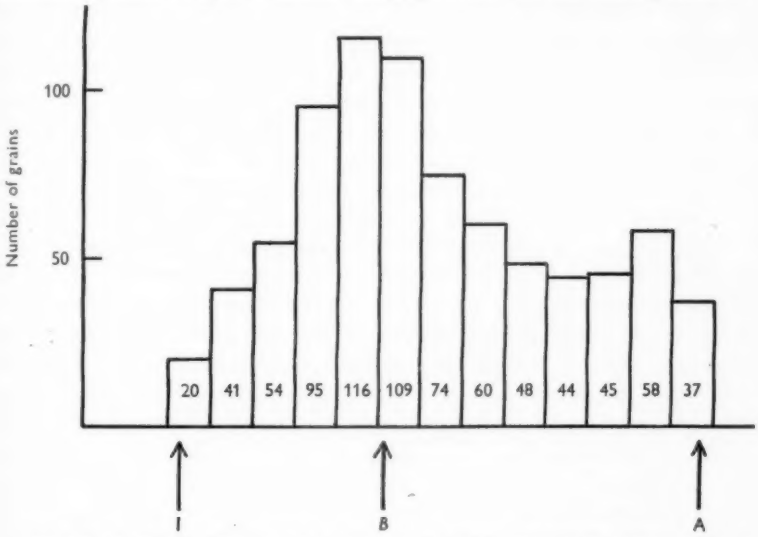
The remaining 30 % of the nucleotide, which is the fraction not located in the N-band region, can be said to be located somewhere in the A-band. Its position cannot be defined with greater precision than that. The rather uncertain indication of a peak near the centre of A is not sufficient to allow an analysis.



Text-fig. 2. Distribution of grains over a section of normal muscle. Area with moderate nucleotide content (71 μc/g). A total of 1979 grains is represented. Sarcomere length 2·5 μ. Muscle section 1·0 μ thick; exposure 21 days.

The use of transverse sections for analysis. One of the objections (Hill, 1959*a*) to the use of oblique or transverse sections for improving the resolution with autoradiography by making use of a spread-out pattern of striations had been in connexion with the difficulty of seeing the striations by polarized light in such preparations. The use of stained sections overcomes this particular obstacle, but the second objection remains, namely that, except in extremely thin sections, oblique cutting results in a confused picture due to overlapping of material from one part of the sarcomere with that from an adjacent one. Sections have to be as thin as 0·1 μ before such overlapping ceases to be of importance. It was decided

not to use this method of improving resolution mainly for two reasons: first, the stain intensity in 0·1 μ sections would probably be insufficient for accurate locations, and secondly the danger of the dispersion of the lanthanum nucleotide increases when the sections are made thinner. The bizarre character of the striation pattern seen in a nearly transverse section, with its great range of band spacings, would also hinder any analysis. The bands in a muscle are seldom at right angles with the long axis, and they are often seen to be curved, so it is not surprising that very small parts of most transverse sections are sufficiently parallel with the striation planes to give a useful 'magnification' of the band pattern.



Text-fig. 3. Distribution of grains over a section of normal muscle. Area with low nucleotide content (22 μc/g). A total of 801 grains is represented. Sarcomere length 2·5 μ. Muscle section 1·0 μ thick; exposure 21 days.

The stain pattern in normal muscle

The effect produced by staining a muscle section through the autoradiographic film (Pl. 2, fig. 1) is sufficient for the purpose of referring the grains to the A and I bands, but it does not show clearly the real form of the stainable material. The stain pattern and its variation under certain conditions has been studied in sections which were not covered with the film. The basic dye crystal violet has been used, and a few experiments have been done with silver nitrate.

The sections were stained through the methacrylate polymer. The permeability of the methacrylate was found to be variable, as judged by

the time required for staining to full intensity. Some blocks prepared on an earlier occasion from the m. ext. long. dig. IV gave sections with a much higher stain permeability than those used for the autoradiographs, and it was found that with these highly permeable sections the stain pattern was changed by prolonging the action of the stain solution or by raising its temperature.

It was found that the permeability of the methacrylate was very greatly decreased by heating the slides to 160–180° C for a few minutes before staining. At 180° C the methacrylate was found to become so impervious that extremely prolonged staining was necessary at room temperature, and the staining was sometimes done at 40–50° C to accelerate it. In sections which had been heat-sealed in this way the stain pattern was of the form shown in Pl. 3, figs. 1, 2. The stain in the I band is in two lines, near the boundary with A. These will be referred to as the N bands, for they probably correspond with the bands so designated by the microscopists of the last century.

The result of staining an unheated section may be quite different, though only in those cases where the methacrylate has a high permeability. The sections from the radioactive muscle were relatively impermeable without previous heating, and though the definite separation of the lines in the I band was generally (though not always, e.g. Pl. 3, fig. 3) obscured by blurring of their outlines, the stain remained in the I band, as in Pl. 2, fig. 1, where there is actually a faint indication of the division into N bands. On the other hand, sections from the block with relatively permeable methacrylate gave the normal pattern only if they had been heated to 160–180° C before staining; when they had not been heated they gave a variety of patterns, some entirely different from the normal, and similar to those seen in the glycerol–water-extracted muscle. The variety of patterns shows stages in a definite sequence, starting with the normal N bands and ending with the pattern seen in Pl. 4, fig. 5. The order in this sequence was fairly obvious, because successive stages can be seen merging, one into the other, in neighbouring sarcomeres, or even in one and the same sarcomere (Pl. 3 figs. 5, 6). The first change is seen as a blurring of the N bands: each band appears to spread in both directions along the sarcomere, so that the light space between the N bands fills up, and their outer boundaries become further apart (bottom of Pl. 3, fig. 5). The next stage involves further longitudinal spread of the stain (Pl. 4, fig. 2); the stain which in the previous stage had filled up the centre of the I band now remains as a sharply defined Z line. The spread towards A is halted at a well defined line just inside the A band. At this stage the non-banded, diffuse part of the stain remains in I. Next (Pl. 4, fig. 3) the non-banded stain has been transferred from I to A. This is followed by

stages (Pl. 4, fig. 4) where there is a progressive loss of the non-banded stain, leaving finally a simple banded pattern, as seen in Pl. 4, fig. 5, consisting of a Z line and two lines just within the boundaries of the A band: the latter will be referred to as 'A lines'. This end-result has been found only in the glycerol–water-extracted muscle. When it occurs it is seen that the muscle has begun to show separation into myofibrils; this suggests that sarcoplasmic elements, now removed, held the non-banded stain.

The movement of the stainable material in a section of the normal muscle occurs only in the presence of the stain itself. Sections of the block which showed the greatest readiness to give the transformed stain pattern were not affected by treatment with a stain-free buffer solution, even at a raised temperature. A section which, before heat-sealing, would have given the pattern of Pl. 4, figs. 2, 3, was immersed in 100 mM pH 4 buffer at 60° C for 8 min. It was then sealed by heating at 185° C for 10 min. It was stained in the usual way; a sharp N-band pattern was obtained. It appears from this that the stainable material is mobilized by the presence of the dye cations, and that the formation of a compound with the dye is necessary to confer mobility on the stainable material.

In transverse sections presenting the N-band pattern the stain intensity is uniform over any individual fibre. This means, in view of what has been found concerning the uneven distribution of *free* nucleotide, that the latter does not stain. It was suggested (p. 350) that a compound with free nucleotide is, in fact, formed by the dye, but that it is washed out of the section either during staining or during the subsequent washing in buffer. This could be tested by making autoradiographs of previously stained sections, to see whether the aggregates of free nucleotide had been removed.

Silver nitrate staining of muscle sections. Dempsey, Wislocki & Singer (1946) demonstrated the argyrophilic properties of regions of isotropic muscle corresponding with the N bands. The double sets of lines reported in the earlier paper (Hill 1959*a*) and attributed to a reaction with the silver in the autoradiographic emulsion probably indicate the same elements in the sarcomere.

Experiments were done to test the reaction of silver nitrate with methacrylate-embedded sections cut from the osmium–lanthanum-fixed muscle. No reaction in the form of a silver deposit could be obtained. It seems likely that this failure to react with silver is due to the presence of lanthanum, which blocks the binding sites, for in a lanthanum-free preparation, fixed by freezing, a good silver reaction was obtained.

Preparation of the muscle for silver staining. To prevent the formation of ice crystals, which were probably responsible for the imperfect preservation of a muscle by freeze-drying in the earlier work (Hill, 1959*a*), tests were

made to see whether the living muscle (m. ext. long. dig. IV) could be equilibrated with glycerol up to 2·0 M in Ringer's solution. At this concentration glycerol would at least greatly reduce the damage by formation of ice. It was shown, however, that even with a very slow increase of the glycerol content of the Ringer's solution a concentration exceeding 1·5 M was toxic. Some benefit, though not perfect preservation, would be likely to result from using a weaker glycerol mixture. A muscle was therefore equilibrated with 1·0 M glycerol, the concentration being raised slowly over a period of 24 hr at 2° C. It was then electrically excitable. It was rapidly frozen at −160° C. In the presence of glycerol the muscle could not be dried in the usual way by low-temperature vacuum evaporation, so the ice and glycerol were removed by ethanol substitution at −80° C over a period of 3 days. The muscle was embedded in methacrylate and sectioned at 1·5 μ. The muscle structure, as seen by polarized light, was found to be relatively well preserved, and definitely better than that seen in muscles prepared without using glycerol. The sections were stained by floating them on an unbuffered solution of silver nitrate (1·47 mM) at 50° C for 3 min. After washing the sections by transference to the surface of pure water, they were picked up on slides and immersed in ordinary photographic developer for 5 min to reduce the silver salts to visible metallic silver deposits.

The picture so produced was variable, and although in most parts of a section a pair of lines in the N band region was discernible it was always superimposed on a background of silver granules which sometimes practically obscured it. A clearer picture was obtained by removing soluble material from the section with hot water before staining with the silver nitrate. The lines of silver deposit in the I bands were thus greatly clarified (Pl. 3, fig. 4).

Although, as with the basic dye, there is little information regarding the identity of the material so revealed, it may be said that bound phosphate is probably a constituent which could be located by this method. Free nucleotide is readily removed from thin methacrylate sections of a muscle fixed by freezing (Hill, 1959*a*); the same is probably true for other free phosphates and for chloride ions. Buffering the silver nitrate at pH 4, which should preclude the formation of silver proteinates through the carboxyl groups, makes no difference to the stain pattern, so binding by protein is not involved.

The argyrophilic sites are arranged in a regular order across the muscle, at a spacing corresponding with the myofibrils, but it is not possible to say whether they lie on the myofibrils or between them. It seems very likely that they correspond with the N bands as seen by staining with the basic dye.

The spacing of the N bands. The polarized light microscope showed clearly that the N bands revealed either by the basic dye or by the silver lie within the I bands, and this was confirmed by measurement. In Pl. 3, fig. 2, the length of the sarcomere is $2{\cdot}7\,\mu$. The A band has a constant length of $1{\cdot}5\,\mu$ (Huxley & Niedergerke, 1958), so the I band is $1{\cdot}2\,\mu$. The separation of the N bands is $0{\cdot}74\,\mu$. In the silver-stained specimen (Pl. 3, fig. 4) the sarcomere length is $3{\cdot}1\,\mu$, the I band is therefore $1{\cdot}6\,\mu$. The spacing of the N bands is $0{\cdot}98\,\mu$. (The blocks were sectioned so that the knife moved at right angles to the long axis; compression of the methacrylate has therefore not interfered with the accuracy of the above measurements.) The dependence of the spacing of the N bands on the over-all sarcomere length is a point of some interest which is discussed below.

The position of the bands in the fully extracted condition (Pl. 4, fig. 5) is also of interest. The lines on either side of the A band have their outer edges $1{\cdot}4\,\mu$ apart and their inner edges $0{\cdot}8\,\mu$ apart, each band being $0{\cdot}3\,\mu$ wide. This shows in the first place that the bands lie entirely within A; secondly, taking the length of the I filaments as $2{\cdot}0\,\mu$ (Huxley, 1957), the amount of overlap of the A and I filaments for the present sarcomere length ($2{\cdot}2\,\mu$) should be $0{\cdot}65\,\mu$, which is more than twice as much as the width of the observed bands. These bands, therefore, do not correspond with the regions of overlap of the A and I filaments, and they must represent some independent element in the A band, adjacent to the A–I boundary.

Autoradiography of the poisoned muscles

In transverse section the autoradiographs (Pl. 1, figs. 2, 3) of the muscle fixed at the peak of a caffeine-induced contracture and of the muscle stimulated to exhaustion after poisoning with iodoacetate and cyanide are essentially similar to those of normal muscle. They show the same type of uneven distribution, with aggregation near the fibre surfaces, which denotes the transverse freedom of diffusion within the fibre. This finding is discussed later.

The longitudinal distribution, with reference to the bands, could not be ascertained, because the striations could not be seen, except in scattered small patches, either by staining or in polarized light. It is not known whether the disorganization responsible for this was caused by the myofibrils having lost transverse register with one another (the result, perhaps, of violent mechanical stresses), or whether some more profound destructive change had supervened. It can only be said that the degradation had not proceeded to the point at which nucleotide had been able to escape from the fibres, for the over-all concentration of isotope was approximately the same as that of the normal muscle.

Autoradiography of the glycerol–water-extracted muscle

Transverse sections. The autoradiographs (Pl. 1, fig. 4) of the extracted muscle show an even distribution of the isotope within the fibres and they are in this respect in striking contrast with those made from sections of the normal muscle. The grain count averaged for several separate areas over a 0·5 μ transverse section was 0·76 grains/100 μ^2.day, corresponding to 19 μc/g. This muscle was taken from frog 2, which had received 167 μc/g, so the yield, scaled to correspond with the injection given to frog 1 (230 μc/g) would be 26 μc/g. It was found that the isotope concentration in the normal muscle was 140 μc/g. Thus the extracted muscle held 19% of the nucleotide originally there. This is higher than the value expected from the carbon-14 experiments with glycerol–water extraction, though it is close to that for the water extraction.

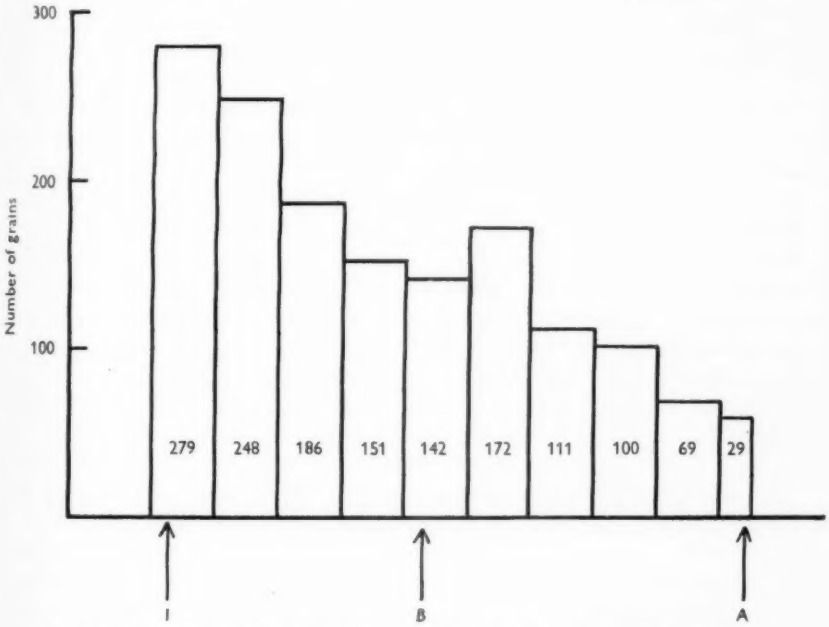
Longitudinal sections. The stain pattern of the extracted muscle in longitudinal section shows that the changes resulting from extraction were incomplete at the time when the muscle was fixed, for the pattern had a variety of forms in different parts of the muscle, within different parts of the same fibre, and even in one and the same sarcomere (Pl. 3, fig. 6). The only stage in the transformation at which it was possible to obtain a sufficient area of section showing a consistent staining reaction was that seen in Pl. 2, fig. 2. The grain yield in this region was 0·97 grains/100 μ^2.day, and for the section thickness of 1·0 μ this corresponds to 18 μc/g, which is about the same as that found from the transverse sections. The analysis of grain distribution is shown in Text-fig. 4. When this is compared with the analysis for normal muscle (Text-figs. 1, 2, 3) it is seen that the isotope has now become distributed in a quite different way. The concentration is now at its peak on the Z line, and there may be a secondary peak in A about at the position which is seen stained in the later stages of extraction (Pl. 4, fig. 5). In the normal muscle, lines only 0·9 μ apart appear to have been satisfactorily resolved as separate sources. In the extracted stain pattern the lines are only 0·7–0·8 μ apart, and the chance of their individual resolution by autoradiography is much reduced, even by this relatively small decrease in separation. There is not, in fact, enough evidence to say whether the new distribution consists, in part at least, of lines of nucleotide aggregation at sites corresponding with the lines seen by staining.

DISCUSSION

Autoradiography

Resting muscle. The analyses show that in a depolarized, osmium-lanthanum-fixed muscle a high proportion, about 70%, of the nucleotide lies in two bands, possibly the N bands, within the isotropic region of the

sarcomere. This proportion is much higher than the 20 % estimated in the earlier experiments (Hill, 1959*a*) where the muscle was fixed by freezing. The muscle structure is well preserved after 'chemical' fixation, and the possibility of dispersion of the nucleotide is reduced by its being bound by lanthanum. From this point of view the present results are the better for having used 'chemical' fixation. On the other hand, this kind of fixation results in an abnormal distribution of nucleotide within the fibre.



Text-fig. 4. Distribution of grains over a section of extracted muscle. Isotope content of muscle, 18 μc/g. A total of 1487 grains is represented. These are grouped in 10 blocks between the centre of the I band (arrow at left), and the centre of A (arrow at right). The arrow at *B* is placed 0·75 μ from A, and represents the position of the A–I boundary. Sarcomere length, 2·7 μ. Muscle section 1·0 μ thick; exposure 23 days.

It is almost certain that this is attributable to the slow penetration of the nucleotide precipitant through the fibre surface, allowing the nucleotide time to diffuse to the site of precipitation. The redistribution might, at first sight, be supposed to have rendered the results too artificial to have any relevance to normal muscle. This is not necessarily so if the analysis is confined to the longitudinal axis, that is with reference to the boundaries of the A and I bands. It is clear that the nucleotide has a transverse

freedom of movement within the muscle fibre, but any preference for siting at particular points on the longitudinal axis is not necessarily affected by allowing transverse movement to occur. This transverse movement is caused by diffusion towards a vacant region created by nucleotide having been rendered insoluble at that point, and one might argue that since the only change encountered by the free nucleotide molecules while this precipitation is proceeding is in the concentration around them of their own species an abnormal, or false, picture would show up in the form of a difference in distribution as between the areas of the fibre where there are ultimately high concentrations of isotope, and those of low concentration. No such difference is found. Actually it is surprising to find that the percentage of the total nucleotide which is aggregated in the N bands should be the same irrespective of whether the area is one of high or one of low concentration. In the regions of lowest density it is likely that the nucleotide is of the bound variety, possessing no transverse mobility before fixation; where there is the highest density practically all the nucleotide had freedom to diffuse before it was precipitated by lanthanum. There is a fundamental difference between the two forms, yet it is not reflected in a difference in their distribution. The same thing was found to be true in the earlier work (Hill, 1959*a*), where the distribution in relation to the bands for a section which had been protected against water was more or less identical with that seen when the water-soluble material had been removed.

The combination of transverse freedom of a large part of the nucleotide with longitudinal restraint is possibly associated with a tubular system of communication. No transverse component of the endoplasmic reticulum has been described, in amphibian muscle, in the N-band region of the sarcomere. It is possible, however, that the facility for transverse diffusion may be provided by the specialized structures described by Porter & Palade (1957), and referred to as 'triads', which lie in the spaces between the myofibrils in the region of the Z line, for there is evidence that the middle elements of the triads are connected together to form a transverse system of communication along the region of the Z line.

Another difference between the present and earlier series of experiments has to be considered. This is the state of polarization of the muscle membrane at the time of fixation. In the earlier experiments the muscle was frozen without prior depolarization; in the present series depolarization was inevitable though the conditions were such that the muscle showed no mechanical change. The difference in the results is conceivably due to the change from the polarized to the depolarized state; it would be necessary to do further experiments with a freeze-dried depolarized muscle to show whether this is so.

The N bands. The nucleotide-rich zones in the I band lie in the position of the N bands. These bands have been described (Retzius, 1890) as being probably formed by rows of regularly aligned granules, though the term 'granules' would now be interpreted as meaning 'aggregations' or 'inclusions', possibly within elements of the sarcoplasmic reticulum. The basophilic material of Pl. 3, figs. 1, 2, 3, shows signs of being divided into aggregates with the same spacing as the myofibrils, and this particulate appearance is most marked with the argyrophilic material of Pl. 3, fig. 4.

It is interesting that some of Caspersson & Thorell's (1942) photographs show the ultra-violet absorbing band to be double; this, also, suggests that there is a concentration of nucleotide in the N-band region.

Dempsey *et al.* (1946) have a number of observations to make on the basophilic and argyrophilic material of striated muscle. By means of a silver method, the N bands are clearly revealed. (These authors point out that the argyrophilic bands may indicate sites of adenine nucleotide, the reaction being due to the result of hydrolytic liberation of the ribose which then reacts with silver.)

Dempsey *et al.* (1946) were not able to explain the apparent contradiction between Caspersson & Thorell's (1942) statement that nucleotide is confined to the I band, and the fact that the basophilic material, as revealed by staining is generally described as being in A. Clavert, Mandel & Jacob (1949), for instance, using a method of staining with basic dyes, show the presence of ribonucleic acid largely within the A band. Some light is thrown on this matter by the results given here; it appears that under conditions which are likely to favour the status of normality by hindering the mobility of diffusible material, basophilia is largely seen in the I band. It is readily mobilized by the stain itself.

The spacing of the N bands is probably dependent on the sarcomere length. Dempsey *el al.* (1946) have also suggested that the spacing of the N bands depends on the length of the muscle, the bands being furthest apart when the muscle is extended and apparently coalescing into a single line in a highly shortened muscle. This is an interesting point, because the N bands are outside the region where the A and I filaments overlap, and they must therefore be drawn to and fro by some unknown connexion with the A band. Elements of the endoplasmic reticulum (Porter & Palade, 1957) may prove to constitute this connexion. In amphibian muscle the reticulum is made up of a mid-A cisterna, connected by longitudinal canalicular elements with the terminal vesicles in the I band. If the N band material lies within the sarcoplasmic reticulum these longitudinal connexions might cause the N-band spacing to vary when the muscle lengthens or shortens; this would depend on the relative extensibility of the various elements of the reticulum. If the elements through A were less

extensible than those connecting the vesicles on one side of the I band with those on the other, the distance between an N band and the boundaries of A would tend to remain constant.

Perhaps the electron microscope will be the means of giving further information on the N-band material and on the structures within which it lies. This will depend on the electron density of the precipitated lanthanum nucleotide. The difficulty may lie in ensuring the retention of lanthanum in water-floated sections of less than $0{\cdot}1\,\mu$ thickness.

Poisoned muscles. The results with the caffeine- and with the iodoacetate-poisoned muscles have been limited to observations on the distribution of the labelled material in transverse sections. It is quite clear that even after extreme metabolic activity the *transverse* mobility of the nucleotide is maintained: there is no evidence of any increase in the binding of nucleotide to structural protein, or to sarcoplasmic structures, under such conditions.

It is worth recalling that Caspersson & Thorell (1942) found a difference in the location of ultra-violet absorbing material in fatigued as compared with resting insect muscle fibres. The sharp outline between the bands seen at the nucleotide-absorbing wave-length was more or less smudged out in the fatigued muscle, and the absorbing material appeared to spread towards the middle of A through a system of fine tubules. In the present experiments the disappearance of organized structure in the poisoned muscles has prevented any analysis being made of the longitudinal location of nucleotide in the fatigued state.

The glycerol–water-extracted muscle. The situation in the glycerol–water-extracted muscle is rather confused because the process of extraction is largely incomplete, as judged by the variability of the stain pattern in different parts of the muscle. It is possible to sort out the sequence of stages in the pattern as the extraction proceeds but it is virtually impossible, with the amount of information available, to say how the over-all concentration, or the distribution of isotope in the sarcomere, changes with each stage. One stage alone (Pl. 2, fig. 2) was sufficiently represented to allow analysis to be done. Here the distribution of isotope was found (Text-fig. 4) to be entirely different from that seen in the normal muscle; the N-band aggregate has been lost and replaced by a Z–A type of distribution. It was pointed out above (p. 366) that in the regions of lowest nucleotide concentration in the normal muscle what remained could be regarded as bound. If this fraction is identical with that remaining after extraction, it appears that it has been shifted by the action of glycerol–water to a new position, at least for the stage which could be analysed. The evidence is too scanty to say whether a parallelism between stain pattern and nucleotide location obtains in general.

The meaning of the stain pattern is in many respects quite obscure. The change from one type of pattern to another can be interpreted either in terms of bodily transposition of the stainable molecules or, alternatively, of the transfer of stain affinity from one set of structures to another by migration, perhaps, of phosphate ions. It has been seen that in some circumstances, when the embedding medium retains a certain degree of permeability, the transformation can be made to occur even in fixed material, and an hypothesis involving movement of large molecules such as those of nucleic acid (even the smaller molecules of ribonucleic acid) would seem to be very far-fetched. Only two points seem fairly clear. First, the N-band stain represents bound and not free nucleotide, for the intensity of staining is constant, and bears no relation with the distribution of the isotope in transverse sections of normal muscle. Secondly, at the other end of the sequence, the most highly extracted portions of the glycerol–water-treated muscle may be assumed to have lost the acid-soluble nucleotide, leaving the ribonucleic acid in the formation seen in Pl. 4, fig. 5. If these ideas are correct it is surprising that one never sees the two patterns together; this may be due to the difference in stain affinity of the N bands, and of the A and Z lines. The N-band material stains more darkly than the A and Z material (this is not noticed in the figures because of the adjustments in contrast during photography), and in addition there is always a generalized staining of the whole sarcomere, though only faintly, under conditions when the N bands are seen. For both these reasons the A and Z lines may be obscured.

At the extreme end of the sequence, with the simple A and Z lines remaining, the myofibrils are in process of becoming separated, and in some parts of the muscle they are entirely free from one another. Under these conditions all sarcoplasmic elements, including the tubular reticulum, have probably been lost. The material which stains must be firmly attached to the myofibrils themselves. Perry & Zydowo (1959), finding ribonucleic acid in well-washed myofibrils, have said, on similar grounds, that it cannot be associated with the sarcoplasmic elements. The apparent parallelism in the loss of the diffuse stain and the loss of the sarcoplasmic constituents is evidence for the view that this diffuse fraction of the stainable material lies either free in the sarcoplasm or in the reticulum. For that reason the mobility of this fraction is not so questionable as that of the fraction (thought to be ribonucleic acid) with undoubted fibrillar rather than sarcoplasmic connexions.

Just as the N bands are interesting in their relationship with the A–I boundary, so likewise are the lines seen in the extreme stages of glycerol–water extraction. The pair of lines within the A band have the significant property of being so narrow that they cannot simply be indicating the

regions of overlap of the A and I filaments. They therefore represent some distinct feature in the A band, the counterpart, as it were, of the N bands which are immediately adjacent to the other side of the A–I boundary.

Ultra-violet dichroism

Measurement of the ultra-violet dichroism of frog's muscle (Hill, 1959*b*), showed that the dichroism associated with adenine nucleotide was such that it would be caused by transverse orientation of less than 10% of the molecules present in living muscle. The remaining 90% could be regarded as 'freely mobile at least within its own sphere (for instance, within a sarcoplasmic vesicle), if not within the fibre as a whole'. This is consistent with the conclusion arrived at here in respect of the transversely mobile nucleotide, which amounts to about 90% of the whole: it clearly has freedom of movement in two dimensions, so its lack of binding to oriented structures precludes any likelihood of its taking up a preferred orientation in relation to the muscle axis.

SUMMARY

1. Tritium-labelled adenine nucleotide was located in sections of the sartorius muscle of the frog by autoradiography. The frog was injected with tritiated adenine, and this was converted into nucleotide within the body.

2. Earlier work on the same problem had been handicapped by the poor preservation of a muscle fixed by freeze-drying. In these experiments osmium tetroxide fixation was used, with lanthanum as the nucleotide precipitant.

3. The muscle sections were stained with a basic dye to enable the striations to be seen clearly, and it was possible to locate the autoradiographic grains with greater accuracy than hitherto.

4. It appears that about 70% of the adenine nucleotide is located in the region of the N bands, within the isotropic part of the sarcomere.

5. Although the nucleotide shows this definite binding in the longitudinal direction, it appears to be free to move transversely across the muscle, and the slow penetration of the nucleotide precipitant during fixation results in accumulation of the isotope near the surfaces of the fibres.

6. The distribution of nucleotide in a muscle extracted with a glycerol-water mixture was also examined.

7. Metabolic exhaustion caused by iodoacetate-cyanide, or by caffeine, so disorganized the structure of the muscle that the longitudinal distribution of nucleotide could not be investigated. It was found that under such conditions the transverse mobility of the nucleotide was maintained.

8. The stain pattern with basic dyes takes on a variety of forms and has proved to be of some interest.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Autoradiograph of a transverse section of a normal tritium-labelled sartorius muscle. Section thickness 1·0 μ. Exposure 22 days. The section is unstained, and to show the location of the isotope at low power the autoradiograph has been over-developed (30 min in Kodak D. 19b), and the degree of shading represents the extent of radioactivity. The isotope, indicating the adenine nucleotide, is unevenly distributed in the individual fibres and this effect is most marked in the fibres near the centre of the muscle. The outer surfaces of the muscle are at the top and bottom of the figure.

Fig. 2. Autoradiograph of a transverse section of a sartorius muscle poisoned with iodoacetate and cyanide, and stimulated to exhaustion. Section thickness 0·5 μ; exposure 35 days. Unstained. The autoradiograph was over-developed (30 min in Kodak D. 19b), and the degree of shading represents the extent of radioactivity in the different regions. The outer surfaces of the muscle are at the top and bottom of the figure.

Fig. 3. Autoradiograph of a transverse section of a sartorius muscle fixed at the peak of a caffeine contracture. Section thickness 0·4 μ. Exposure 27 days. Unstained. The autoradiograph was over-developed (30 min in Kodak D. 19b). The outer surfaces of the muscle are at the top and bottom of the figure.

Fig. 4. Autoradiograph of a transverse section of a sartorius muscle extracted in glycerol–water before fixation. Section thickness 0·5 μ. Exposure 35 days. Over-developed for 30 min in Kodak D. 19b. Unstained. The grain yield, representing the nucleotide concentration, is uniform within the individual fibres.

Plate 2

Fig. 1. Autoradiograph of a longitudinal section of the tritium-labelled normal muscle. Section thickness 1·0 μ. Exposure 21 days. Section stained with crystal violet, showing the I bands dark.

Fig. 2. Autoradiograph of a longitudinal section of the glycerol–water-extracted muscle. Section thickness 1·0 μ. Exposure 23 days. Section stained with crystal violet, showing the Z line and the A band dark.

Fig. 3. Higher-power photograph of part of the specimen shown in Pl. 1, fig. 1; the aggregation of nucleotide near the surfaces of the fibres is very marked.

Fig. 4. Higher-power photograph of part of the specimen shown in Pl. 1, fig. 1. These fibres are near the surface of the muscle, and the aggregation of nucleotide near the surface of the fibre is less marked than it is in fig. 3.

Plate 3

Fig. 1. Section of a normal m. ext. long. dig. IV fixed in the osmium–lanthanum mixture. The section was heat-sealed at 185° C for 10 min, and stained with crystal violet. Sarcomere length, 2·63 μ.

Fig. 2. Section of a normal m. ext. long. dig. IV fixed in the osmium–lanthanum mixture. The section was heat-sealed at 190° C for 13 min, and stained with crystal violet. Sarcomere, 2·73 μ.

Fig. 3. Section of a normal sartorius muscle, fixed in the osmium–lanthanum mixture. The section was not heat-sealed before staining with crystal violet.

Fig. 4. Section of a freeze-dried m. ext. long. dig. IV stained with silver nitrate. The pairs of lines lie within the I bands. Sarcomere, 3·14 μ.

Fig. 5. Section of a glycerol–water-extracted sartorius muscle, cut at 45° to the axis. Section thickness, 0·4 μ. Stained crystal violet. This specimen was selected to show how the stain pattern varies within an individual fibre of the extracted muscle. At the bottom the stain is in the I band; at the top, the Z line and the A lines are stained.

Fig. 6. Section of a glycerol–water-extracted sartorius muscle. Section thickness, 0·8 μ. Stained crystal violet. This specimen was selected to show how the stain pattern may vary within one and the same sarcomere. On the right the whole of the I band is stained; on the left the stain is seen only in the Z line and in the A lines.

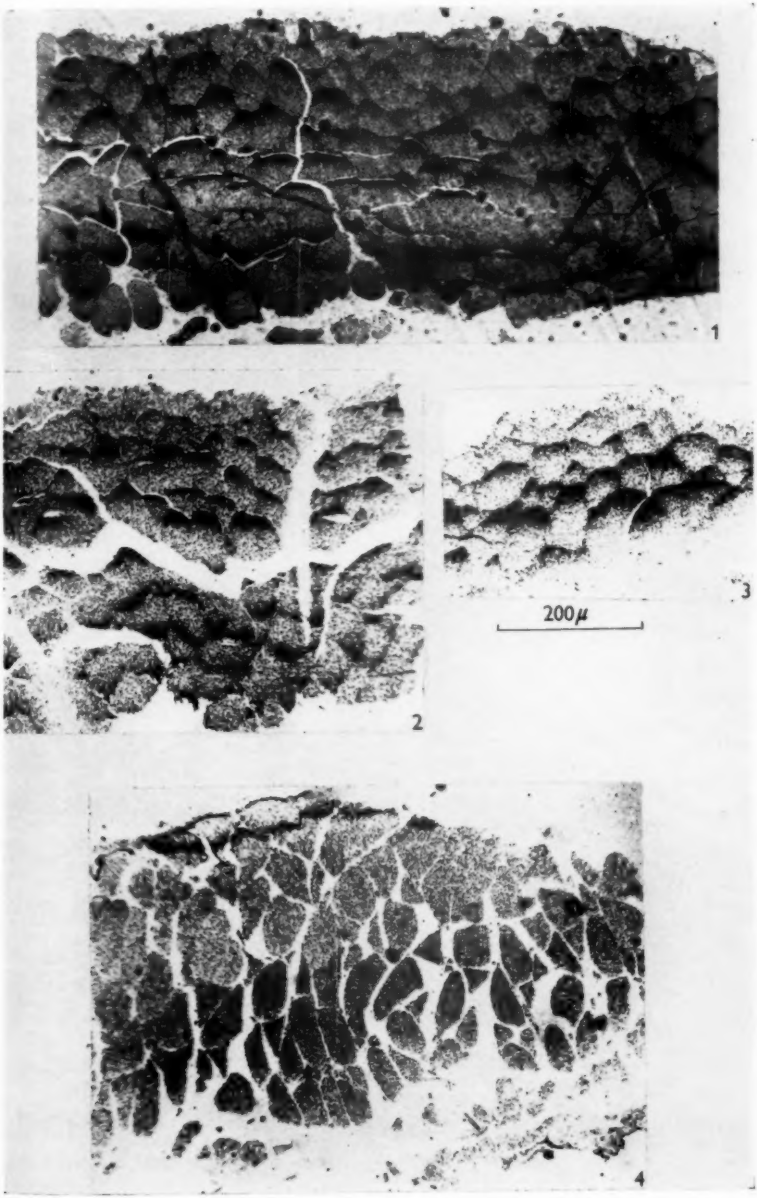
Plate 4

Fig. 1. Section of the extracted muscle cut at 45° to the axis. Section thickness, 0·4 μ. Stained crystal violet. The stain pattern is at the stage where the stain in I is spreading towards A, and the Z line has become prominent. The example is selected to show the honeycomb-network of channels between the Z line and the A lines.

Fig. 2. Section of a m. ext. long. dig. IV, 0·8 μ thick, stained in crystal violet. The methacrylate in this section showed high permeability, and it was not heat-sealed before staining. The stain is no longer in the N bands (compare Pl. 3, fig. 1), but prominent Z and A lines have formed. The non-banded part of the stain is still in the I band.

Fig. 3. Another part of the section shown in Fig. 2. The non-banded stain is now present in the A band.

Fig. 4. Section of the extracted sartorius muscle. Section thickness, 0·8 μ. Stained crystal violet. On the right the Z and A lines are seen, together with the non-banded stain in A.



D. K. HILL

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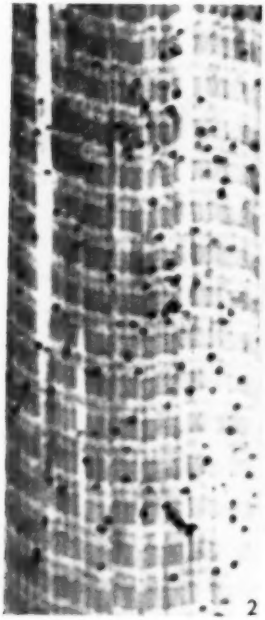

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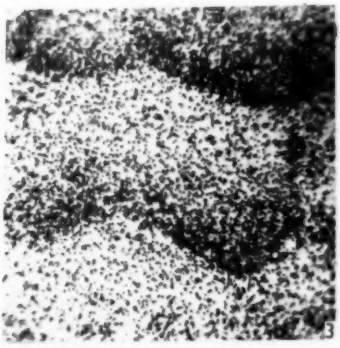

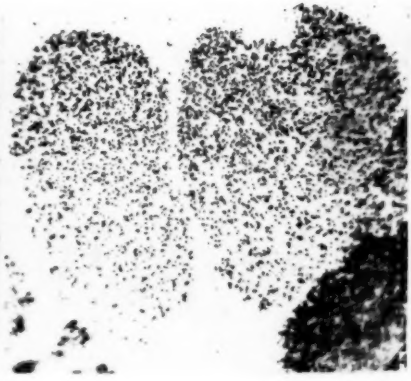

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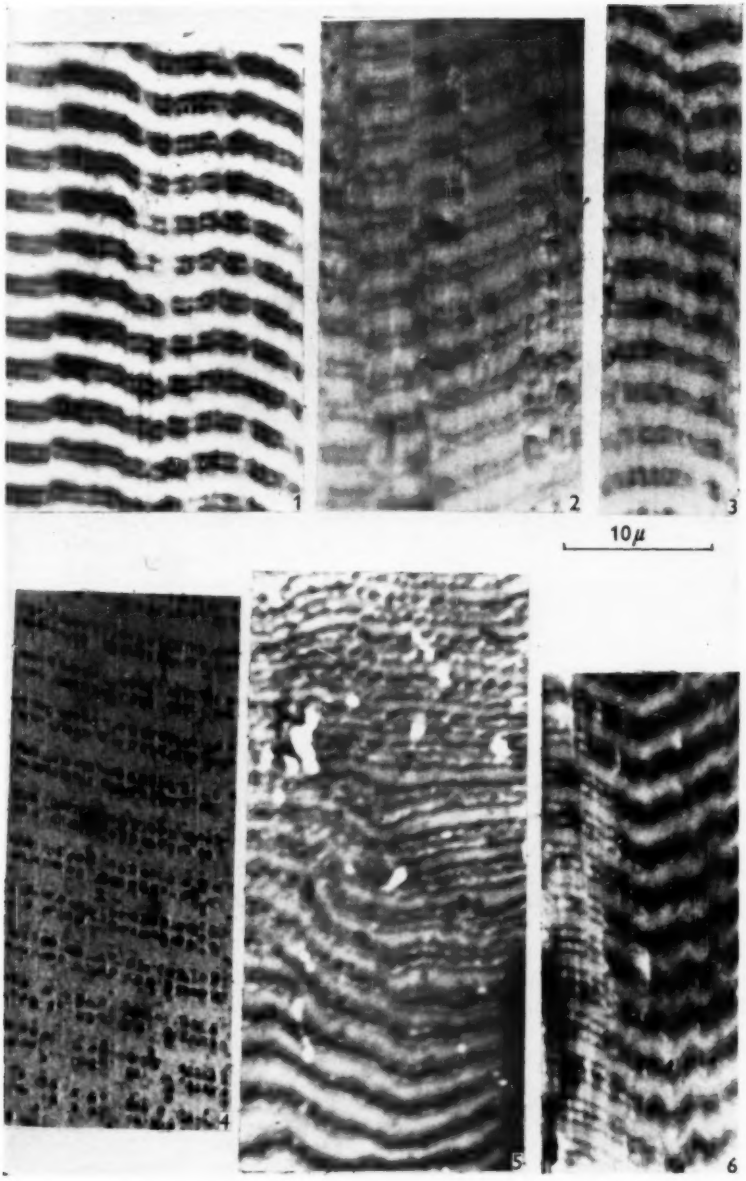

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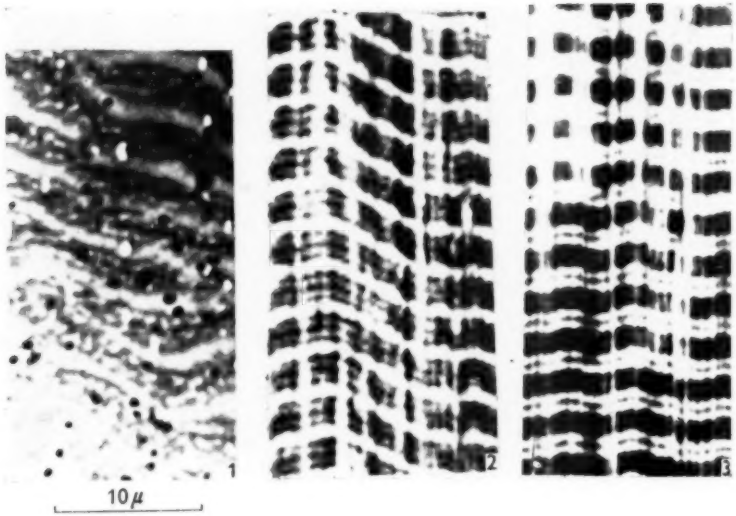
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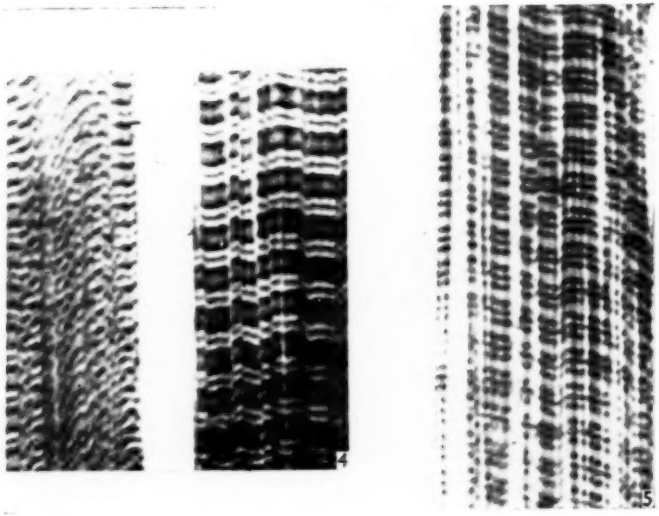
4
5

On the left, in an adjacent fibre, the non-banded stain has been lost, but the striations are confused because the myofibrils have lost register with one another.

Fig. 5. Section of the extracted sartorius. Section thickness, 0·2 μ. Stained crystal violet. The final stage in the transformation of the stain pattern is seen here, with the stain present almost entirely in the Z and A lines. At this stage the myofibrils are becoming separated, and in the greater part of such a section the striations of the myofibrils are out of register with one another, as is seen in Fig. 4.

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SYNAPTIC ACTION DURING AND AFTER REPETITIVE STIMULATION

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Under natural conditions synapses are activated by trains of impulses that may be of relatively high frequency. For example the annulo-spiral endings of muscle spindles often discharge impulses at frequencies as high as 200/sec (Matthews, 1933; Hunt & Kuffler, 1951; Eldred, Granit & Merton, 1953; Granit, 1955); and in conditions of extreme stress with activation by the gamma efferents the discharge frequency may be much higher (Matthews, 1933; Eldred *et al.* 1953) so that synapses may be activated at frequencies up to 500/sec. Presumably it is functionally desirable that such high frequencies should result in a greater rate of output of transmitter substance, the higher frequency of receptor organ discharge resulting in a greater synaptic excitation of motoneurones (cf. Granit, 1955). Repetitive synaptic activation of motoneurones has been studied by the monosynaptic reflex discharges generated thereby (Hagbarth & Naess, 1951; Eccles & Rall, 1951*a*; Jefferson & Schlapp, 1953; Alvord & Fuortes, 1953; Fuortes & Hubel, 1956; Evanson, 1956; Lloyd & Wilson, 1957; Lloyd, 1957*a*, *b*) and by the synaptic potentials recorded either extracellularly or after electrotonic spread to the ventral root (Eccles, 1946; Eccles & Rall, 1951*a*). In the present investigation repetitive synaptic activation has been studied in detail by intracellular recording from motoneurones. An investigation of the response to a second volley over a wide range of intervals leads on to a study of a wide range of frequencies of activation.

There have been many investigations of the changes in synaptic efficacy after single and repetitive stimulation (cf. Hughes, 1958). For example, there have been comprehensive studies by Bernhard (1947), Lloyd (1949, 1952), Ström (1951), Eccles (1946), Brooks, Downman & Eccles (1950), Brock, Eccles & Rall (1951), Eccles & Rall (1951*b*), Beswick & Evanson (1955*a*, *b*) and Granit (1956) of changes signalled by the sizes either of monosynaptic reflexes or of the extracellular synaptic potentials in the spinal cord. By employing intracellular recording it has been possible to

achieve a more analytical investigation of this post-activation potentiation or depression. Intracellular recording of post-activation potentiation of monosynaptic activity has already been reported for standard conditioning tetani (Eccles, 1953; Eccles, Krnjević & Miledi, 1959). However, this present investigation has concerned the post-activation changes induced by a wide range of frequencies and durations of conditioning synaptic stimulation, from single stimuli to high frequencies of 30 sec duration.

Furthermore, an attempt has been made to relate the changes in synaptic activity during repetitive stimulation to the changes observed post-tetanically. In the light of this information the effect of repetitive stimulation on the mobilization of transmitter substance will be discussed. Since the magnitude of post-synaptic potentials, particularly their rate of rise, is a measure of the action exerted by the synaptic transmitter (Eccles, 1957; Curtis & Eccles, 1959), the present experiments give evidence directly relating to the amount of synaptic transmitter released under the varying circumstances of the investigations.

METHODS

All experiments were performed upon motoneurones located within the lumbosacral segments of cats lightly anaesthetized with pentobarbital sodium. The spinal cord was divided in the lower thoracic region. The general methods were those in standard use in this laboratory (cf. Brock, Coombs & Eccles, 1952; Eccles, Fatt, Landgren & Winsbury, 1954; Coombs, Eccles & Fatt, 1955*a*). The intracellular responses were recorded by means of glass micro-electrodes filled with 0·6 M-K_2SO_4 solution. Various muscle nerves were mounted on stimulating electrodes in a warmed paraffin pool. The stimuli were invariably above maximal for Group Ia afferent fibres, and were several times maximal during the long conditioning tetani. Except in special cases, excitatory post-synaptic potentials have been evoked by afferent volleys in the nerve supplying the muscle that is innervated by the motoneurone under investigation. The ventral roots of the L6, L7 and S1 segments were cut. The frequencies of the stimuli used were checked against a standard oscillator and were accurate to ±5%.

RESULTS

Synaptic activation by a second volley

As is illustrated by the intracellular records of Fig. 1*A*, the monosynaptic EPSP's (excitatory post-synaptic potentials) set up by two afferent volleys in the same muscle nerve do not sum until the volley interval is less than about 20 msec. At shorter intervals the size of the second EPSP may be determined by subtracting the initial control response from the summed response. This procedure is justified because an analysis of the summed EPSP's produced by two different volleys converging on the same motoneurone has revealed that the second EPSP suffers a negligible depression on account of the superposition (Eccles, Eccles & Lundberg, unpublished

observations). In Fig. 1*B* the EPSP produced by a second volley in the same afferent fibres was a little larger than the control for volley intervals up to 25 msec. Usually the second EPSP was depressed at all brief intervals (Fig. 2*A*, open circles; cf. Eccles, 1946), this depression passing off with volley intervals in excess of 1 sec. There is a corresponding depression of a testing monosynaptic reflex (Bernhard, 1947; Brooks *et al.* 1950; Brock *et al.* 1951). However, depression of the EPSP was usually less with volley intervals briefer than 50 msec (Fig. 2*A*), there being thus a relative potentiation corresponding to the phase of considerable potentiation displayed with other motoneurones (Figs. 1*B*, 2*B*). At volley intervals from 50 msec to 1 sec depression was regularly observed.

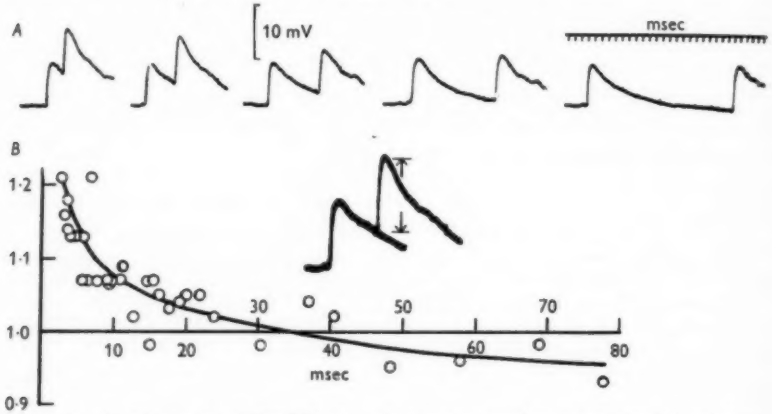

Fig. 1. *A*. Monosynaptic EPSP's set up in a gastrocnemius motoneurone (resting potential, −74 mV) by two maximal Group Ia volleys in the gastrocnemius nerve. The second stimulus was several times maximal strength for Group Ia in order to be maximal early in the refractory period; hence the polysynaptic waves on declining phase of its EPSP. Note calibration scale for d.c. amplifier and time in milliseconds. *B*. Plotting of relative size of EPSP's evoked by second volley against volley interval for series partly illustrated in *A*. Measurement of size of second EPSP is shown in inset (higher amplification than *A*).

When synaptic transmission was blocked by curare, a comparable phase of potentiation (usually 15–30 %) for a second synaptic potential was found in the cat stellate ganglion (Eccles, 1943), and in the turtle superior cervical ganglion (Laporte & Lorente de Nó, 1950). In the absence of curarization block a second volley caused an increased discharge from the cat stellate ganglion for volley intervals as long as 2 sec (Larrabee & Bronk, 1947), while at shorter intervals of activation (up to 0·3 sec) it was shown that a potentiated synaptic action was superimposed on the depression of ganglion cells resulting from their discharge to the conditioning preganglionic volley (Job & Lundberg, 1953).

Mammalian neuromuscular transmission provides an even closer parallel to the findings of Figs. 1, 2. At intervals up to several seconds the second volley sets up a diminished (range 60–90 % of control) end-plate potential (Eccles, Katz & Kuffler, 1941; Liley & North, 1953; Lundberg & Quilisch, 1953); but with volley intervals briefer than 100 msec there is

often a relative potentiation above this depression (Liley & North, 1953), which may even be large enough to give an absolute potentiation (Lundberg & Quilisch, 1953; Hubbard, 1959), just as in Figs. 1*B*, 2*B*.

Thus the response evoked by the testing nerve impulse reveals that activation of both the monosynaptic and mammalian neuromuscular synapses is followed by two opposed processes: a briefer phase of enhanced action is superimposed on a more prolonged depression; and the relative

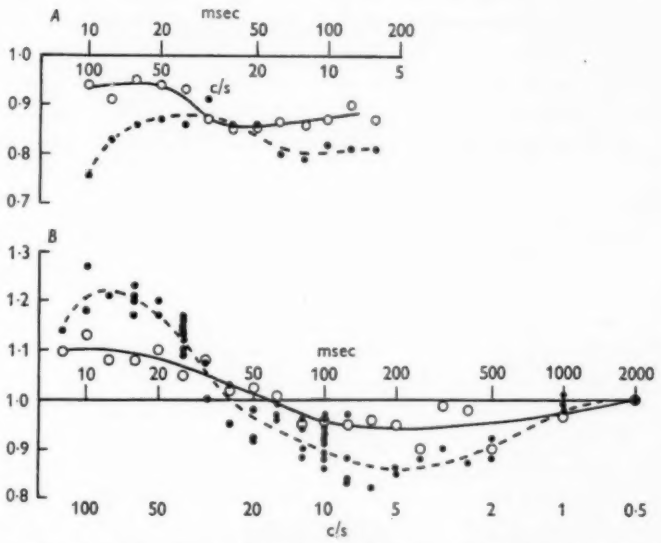

Fig. 2. *A* and *B* are respectively biceps-semitendinosus and gastrocnemius motoneurones with resting potentials of −65 mV and −78 mV. ○, size of second EPSP against volley interval, as in Fig. 1*B*, except that the abscissa scale is logarithmic (note upper scales in milliseconds). ●, relative sizes of EPSP's during the steady state obtaining after the first few responses at various frequencies of stimulation (cf. Fig. 3), as shown by the lower logarithmic scales in cycles per second, the two logarithmic scales being in fact identical. Note that in both *A* and *B* there is a considerable degree of correspondence between the curves for the second EPSP and the repetitive series of EPSP's.

intensities of these two processes vary considerably in different preparations, so that one or the other may be dominant at brief intervals. Potentiation is much more prominent after a single activation of the amphibian neuromuscular junction, particularly when it is curarized (Schaefer & Haass, 1939; Feng, 1941; Eccles *et al.* 1941; del Castillo & Katz, 1954*a*, *b*), but probably it does not differ qualitatively from the potentiation at mammalian synapses.

Repetitive synaptic stimulation

Repetitive stimulation evokes standard responses in those motoneurones in which the monosynaptic EPSP's produced by maximum Group Ia volleys are virtually uncontaminated by superimposed polysynaptic EPSP's or IPSP's. When the temporal summation of EPSP's does not evoke the discharge of impulses, a steady state is attained after the first few EPSP's, even over a wide range of frequencies (Fig. 3; cf. Eccles & Rall, 1951*a*); and this steady state is maintained for hundreds of responses.

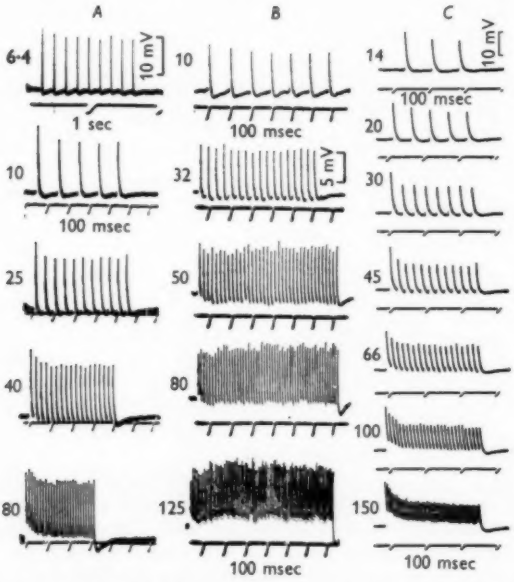

Fig. 3. *A*, *B*, *C* show repetitive monosynaptic EPSP's (intracellular) evoked in biceps-semitendinosus (resting potential, −65 mV), gastrocnemius (resting potential, −78 mV) and deep peroneal (resting potential, −70 mV) motoneurones respectively; frequencies of stimulation are marked on each record. Time scales are in 100 msec, except for scale in seconds on top record of *A*; d.c. amplification throughout. Note potential scales for *A*, *B* and *C*.

When the frequency is below 50/sec, there is virtually no overlap of the successive EPSP's, and it is usually observed that there is a progressive decline in size over the first few responses until the uniformly depressed size is attained (Fig. 3*A*, *C*). It will be seen in Fig. 3 that following each EPSP there is a prolonged after-hyperpolarization which sums during the repetitive stimulation (Brock *et al.* 1952; Coombs, Eccles & Fatt, 1955*b*). With higher frequencies the second and subsequent EPSP's are, in addi-

tion, superimposed on the residuum of preceding EPSP's. As a consequence these EPSP's may attain a higher summit than the first, even though individually depressed in size (Fig. 3*A* at 80/sec). In Fig. 3 the plateaux of both the onsets and the summits of the individual EPSP's have reached a steady value within 200 msec. In different preparations the plateau may be reached, as in Fig. 3*A*, *C*, after an initial phase of rise and decline; or there may be a continued rise to the plateau (Fig. 3*B* at the higher frequencies). In part this initial phase is attributable to summation of the earliest EPSP's, but it must also arise on account of the initial period of adjustment of the EPSP to the steady-state size characteristic of that frequency, and there is also the background of summed after-hyperpolarizations. Two procedures have been adopted in order to measure accurately the sizes of EPSP's during the steady-state response to repetitive stimulation.

The first method is applicable only during the steady state and underestimates the size with those frequencies giving appreciable summation of successive EPSP's, i.e. above 50/sec. If the sweep speed is sufficiently high, sweeps can be repeated at frequencies up to 100/sec, so giving superimposed traces of EPSP's. By a suitable delayer device the frequency can be set at as low a level as one pleases. In this way the superimposed traces of EPSP's were obtained over a wide range of frequencies (cf. Fig. 4*A*). With frequencies of 1/sec or faster the camera shutter was opened after the first few responses in order that all the superimposed traces would occur during the steady state. With low frequencies there were five superimposed traces, but the number was much larger for the higher frequencies. When the sizes of the superimposed EPSP's of the whole series partly illustrated in Fig. 4*A* were plotted against frequency or volley interval (scaled logarithmically), the points on the extreme right reveal that there was no appreciable change in size of EPSP's until the frequency was in excess of 0·4/sec (Fig. 4*B*). There was a progressive depression as the frequency was raised to 5–10/sec. With further increase in frequency, the EPSP increased to a maximum at about 50/sec, being then almost as large as at the lowest frequencies. The decline of the individual EPSP's at still higher frequencies would be in part attributable to superposition on the preceding EPSP's (cf. Fig. 3*B*, at 125/sec). A curve similar to Fig. 4*A* was obtained if the steepest rising slope of each EPSP was measured instead of its height.

This investigation of the sizes of EPSP's during the steady state over a wide range of frequencies has been applied to fourteen motoneurones. The curves so obtained corresponded to Fig. 4*B* in general features; but, as in Fig. 5*A*, the size at the optimal high frequency response was sometimes much below the control level at the lowest frequencies, though with three

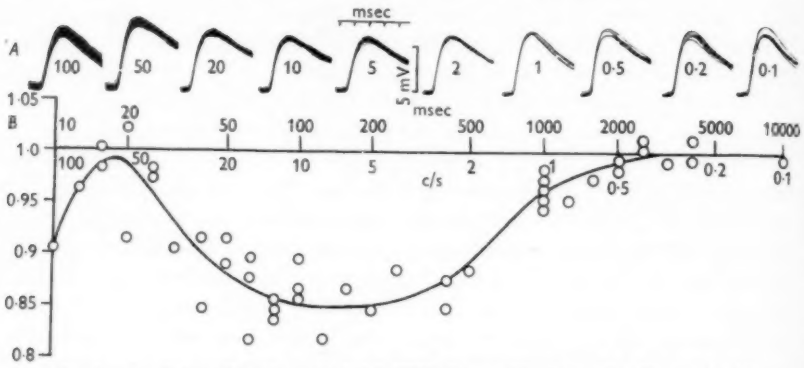

Fig. 4. *A*. Intracellularly recorded EPSP's from a biceps-semitendinosus motoneurone with a resting potential of −62 mV; the superimposed traces were obtained during the steady state of repetitive responses at the indicated frequencies per second. *B*. The EPSP's partly illustrated in *A* are expressed as fractions of the mean size obtaining at 0·4 c/s or slower and plotted against the respective stimulus frequencies on a logarithmic abscissal scale as in Fig. 2. Above the frequency scale the corresponding stimulus intervals are shown in milliseconds.

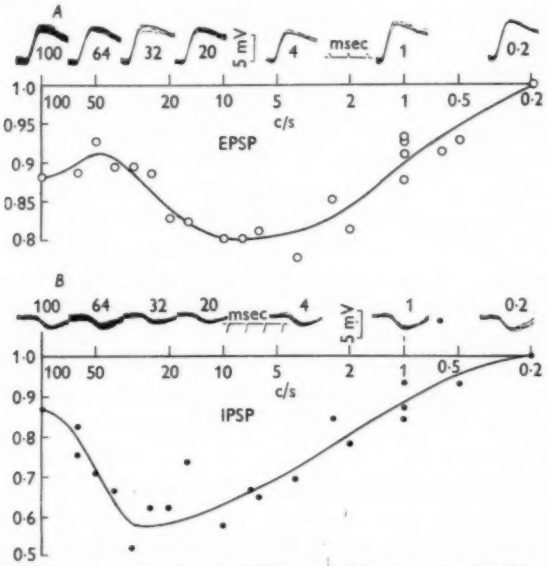

Fig. 5. Plotting as in Fig. 4 of repetitive EPSP's (*A*) and IPSP's (*B*), intracellularly recorded from a biceps-semitendinosus motoneurone with resting potential of −72 mV. Above each series are shown representative records formed by superimposed traces, as in Fig. 4*A*, and at the frequencies indicated in cycles per second. The EPSP's and IPSP's are evoked by maximum Group Ia volleys in biceps-semitendinosus and quadriceps nerves respectively.

motoneurones it was larger. Invariably there were depressed EPSP's at the intermediate frequencies, as in Figs. 4, 5*A*, the range being from 70 to 85% of the control at frequencies in the range of 4–20/sec. The range of the maximum at higher frequencies was from 77 to 128%, the optimal frequency lying between 30 and 100/sec. Repetitive EPSP's from the experiment with one of the largest potentiations are illustrated in Fig. 3*B* and plotted in Fig. 2*B* (filled circles). There is depression at 10/sec, 32/sec is transitional and at 50/sec and 80/sec there is a large potentiation of the EPSP's after the initial period of adjustment.

When EPSP's have been investigated both with double volleys and during the steady state to repetitive stimulation, there was a good correlation between these two responses. For example, a second volley evoked a potentiated EPSP at intervals less than 50 msec in Fig. 2*B* (open circles), and correspondingly the EPSP's evoked at frequencies of 40–100/sec were potentiated well above the control level (Figs. 2*B*, 3*B*). On the other hand, when the potentiation of the second of two EPSP's at brief intervals was inadequate to overcome the depression (Fig. 2*A*, open circles), depression also dominated the repetitive response at high frequencies (Fig. 3*A*, Fig. 2*A* filled circles).

A similar investigation has been performed for the IPSP's set up in two motoneurones by Group Ia afferent impulses (direct inhibition). There was in both a decline with rising frequency to a minimum at 10–30/sec and an increase at still higher frequencies (cf. Fig. 5*B*). Since there is an interneurone on the inhibitory pathway, the frequency–response curve may be significantly modified by this interpolated synaptic relay, temporal facilitation at this synapse contributing at least in part to the increased IPSP at higher frequencies (cf. R. M. Eccles & Lundberg, 1958). Nevertheless, the general similarity of the curves does suggest that inhibitory synapses are affected by the frequency of activation in much the same way as excitatory synapses. The large depression at slow frequencies in Fig. 5*B* contrasts with the finding that under such conditions there is little or no depression of reflex inhibition (Beswick & Evanson, 1957; Wilson, 1958).

In the second method the size of the EPSP during the steady state of the repetitive response (cf. Fig. 3) was determined by expanding the end of the repetitive response at a fast sweep speed (Fig. 6*A*). It can be assumed that during the steady state the decline of the penultimate EPSP of the tetanus follows the same time course as the last; hence by subtraction of this assumed curve the size of the last EPSP can be determined. This method can be applied at high frequencies (Fig. 6*A*), and reveals that the apparent size of the added EPSP declines with increasing frequency, particularly beyond 220/sec. The relationship is best expressed by plotting this size against the stimulus interval, as in Fig. 6*B* (filled circles). There is

approximately a direct proportionality between size of EPSP and stimulus interval for frequencies in excess of about 300/sec and up to 660/sec, which was the highest level applied in our tests. A similar result has been reported for synaptic potentials electrotonically conducted to the ventral root (Eccles & Rall, 1951*a*). The relationship of direct proportionality indicates that the rate of output of transmitter reaches a maximum with

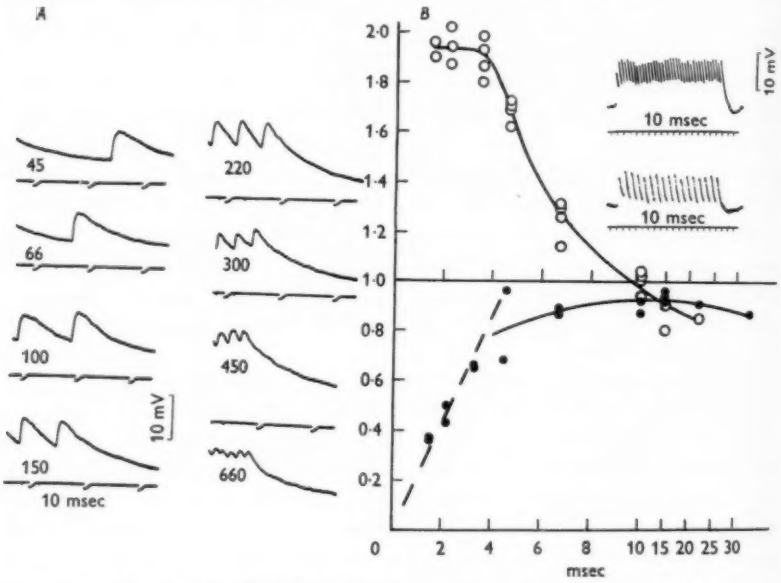

Fig. 6. *A*. Repetitive EPSP's of a gastrocnemius motoneurone recorded intracellularly, resting potential, −68 mV. The records show the end of a repetitive response at the indicated stimulus frequency, which was applied for 170 msec, so that a steady state of repetitive response was attained (cf. Fig. 3); d.c. amplification at the indicated potential scale; time in 10 msec. *B*. ●, sizes of the terminal responses of the repetitive series partly illustrated in *A*, measured relative to the mean value of EPSP's after rest periods of several seconds; the straight line through zero origin shows that there is approximately a direct proportionality at high frequencies. ○, plot of plateau height against stimulus interval for the repetitive EPSP series of the same motoneurone. Inset shows specimen records at 150 and 300/sec.

frequencies in excess of 300/sec. The attainment of a maximum rate of output independent of frequency is also indicated by the curve obtained by plotting the heights of the steady-state plateau against the stimulus intervals (Fig. 6*B*, open circles). There is a rapid increase in plateau height as the frequency rises from 100 to 300/sec, but virtually no change from 300 to 660/sec.

Correlation with the changes in monosynaptic reflexes during repetitive stimulation is only feasible at relatively low frequencies, because at frequencies above 10/sec each successive reflex response is depressed by the after-hyperpolarization that follows a motoneuronal discharge (Brock *et al.* 1952; Coombs *et al.* 1955*a*). At slower frequencies there is good agreement between the two types of investigation. For example, Jefferson & Schlapp (1953) and Evanson (1956) found that reflex depression occurred with frequencies above 0·3/sec, and progressively deepened as the frequency was increased. By employing very sensitive techniques Lloyd & Wilson (1957) and Lloyd (1957*b*) have been able to detect slight depressions with frequencies as low as 0·1/sec. Again, they found increasing depression as the frequency was increased up to 10/sec. After the onset of any frequency of repetitive stimulation a steady state of reflex depression was rapidly attained, just as has been observed for the EPSP's (cf. Fig. 3). At still higher frequencies there was a further deeper depression of reflex discharge, which on experimental evidence was attributed to after-hyperpolarization of the discharging motoneurones. However, at still higher frequencies of activation (usually 60–150/sec, but even as low as 20/sec), there was a phase of relatively less depression, the higher the frequency the less the depression (Alvord & Fuortes, 1953; Lloyd, 1957*a*, *b*). Doubtless much of this effect is due to temporal summation of EPSP's, as suggested by these investigators, but part would also arise on account of the potentiation of EPSP's which contributes to the increased rate of transmitter output for frequencies in excess of 30/sec (Figs. 2*B*, 3*B*, 4, 5*A*). This latter mechanism can be revealed only by intracellular recording.

Repetitive responses of the mammalian neuromuscular junction have been dominated much more by depression (Hutter, 1952; Lundberg & Quilisch, 1953; Liley & North, 1953). However, an effective potentiation was observed when the end-plate potential was depressed by low Ca or high Mg (Lilley, 1956*b*). Potentiation has been a dominating feature with repetitive activation of the amphibian neuromuscular junction (Feng, 1941), particularly during treatment by high magnesium and low calcium (del Castillo & Katz, 1954*b*). Under these conditions there was such a prolonged period of progressive potentiation that a ceiling was still not attained after 50 responses. Since the miniature end-plate potentials showed no increase in size, it was concluded that the potentiation was due to progressive increase in the number of quanta of transmitter emitted by the successive impulses. In contrast, during repetitive activation of normal and curarized amphibian junctions the initial phase of potentiation rapidly gave place to depression of the end-plate potentials, this effect again being due to a corresponding change in the number of quanta emitted by an impulse (del Castillo & Katz, 1954*b*).

Synaptic potentiation and depression after a conditioning tetanus

Recently (Eccles *et al.* 1959) there has been a description and illustration of post-tetanic potentiation of monosynaptic EPSP's after standard conditioning tetani of 400/sec for 10 sec. The present investigation explores the effect of variations in the frequency and duration of the conditioning tetanus. When the post-tetanic potentiation caused the EPSP to generate a spike potential, the steepest part of the rising phase was employed as a measure of the intensity of synaptic action. Probably this is more satisfactory than the height of the EPSP, but this latter measurement has been more convenient, so it has been plotted for those series uncomplicated by spikes. Since there was usually a considerable prolongation of the rising phase of the potentiated EPSP (cf. Eccles *et al.* 1959), larger relative values were obtained for potentiations measured by heights than by slopes.

With increase in the duration of the conditioning tetanus there was at first an increase in both the height and the duration of post-tetanic potentiation; but a ceiling was reached for the height of the potentiation (at 640/sec for 10 sec in Fig. 7*B*), and conditioning tetani of longer duration (*C*, *D*) merely slowed the rise and decline of the potentiation. The potentiations of the EPSP thus provide an exact parallel to the potentiations of the monosynaptic reflex discharges (Lloyd, 1949, Fig. 9). Similar observations have been made on the post-tetanic potentiation both of synaptic

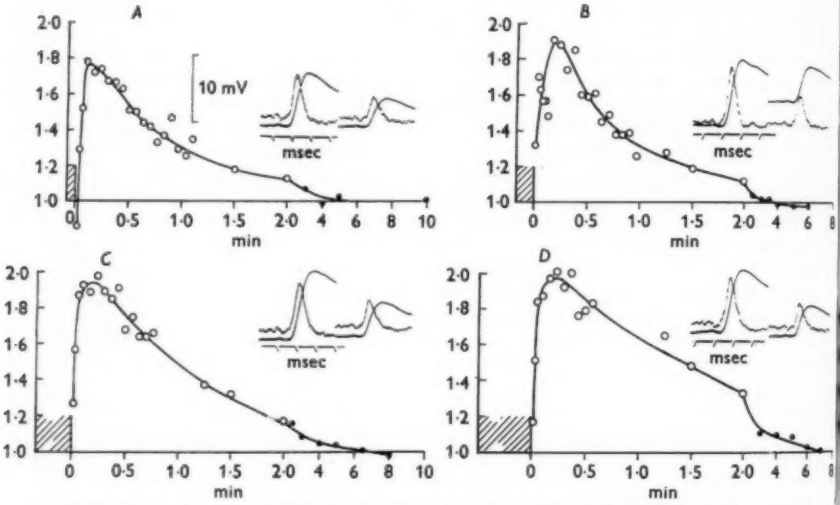

Fig. 7. Post-tetanic potentiation curves of monosynaptic EPSP's of a biceps-semitendinosus motoneurone, resting potential −62 mV. The conditioning tetani were uniformly at 640/sec, the durations being for *A* 5, *B* 10, *C* 20 and *D* 30 sec. Inset records show the EPSP at maximum potentiation and at the initial control level together with the electrically differentiated record. Sizes of EPSP's are calculated relative to the initial mean level which was at least 10 min after a previous tetanic conditioning; durations of the tetani are shown by the initial hatched blocks. Time scale was greatly compressed after 2 min and the symbols were altered to filled circles. All points except those on the rapid rising phase are the means of three consecutive testing responses.

transmission through a sympathetic ganglion (Larrabee & Bronk, 1947) and of mammalian end-plate potentials (Liley & North, 1953). The maximum potentiation of the EPSP was usually in the range 1·5–2·0 times the control (Figs. 7, 8, 10; Eccles *et al.* 1959). Much larger potentiations are usually displayed by reflexes (Lloyd, 1949), because the changes in synaptic efficacy are sampled by the response of a population of motoneurones and not directly, as when the size of the EPSP is employed as the criterion.

When the number of stimuli in the conditioning tetanus was held constant, but the frequency was varied, the maximum potentiation was lower when the frequency was below a critical level of about 300/sec (Fig. 8*B*; cf. Lloyd, 1949). After the lower conditioning frequencies the potentiation followed a slower time course with a later summit and slower decline (Fig. 8*C*, *D*). In these respects also the post-tetanic potentiation of the EPSP parallels the potentiation of monosynaptic reflexes (cf. Lloyd, 1949, Figs. 11, 12). However, potentiation of EPSP's was not usually observed for conditioning frequencies much below 100/sec even when continued for several seconds, though Lloyd reported slight post-tetanic potentiation of reflex discharges after conditioning tetani of 50–75/sec.

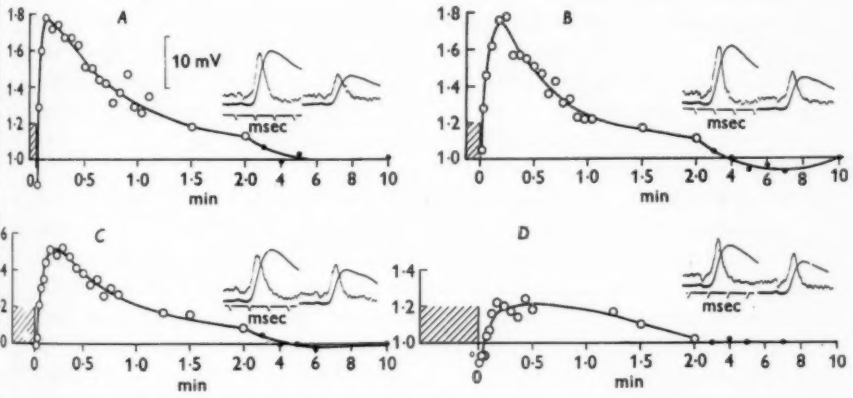

Fig. 8. Series as with Fig. 7 and for same motoneurone, but the conditioning tetanus was in every case 3200 volleys, *A* at 640/sec for 5 sec, *B* 400/sec for 8 sec, *C* 200/sec for 16 sec and *D* 100/sec for 32 sec.

With the mammalian neuromuscular junction there was no potentiation after repetitive activation at 50/sec or lower and 350/sec was adequate for maximum potentiation (Liley & North, 1953).

When post-tetanic potentiation was sampled by a brief repetitive stimulation, there was a rapid decline in the relative potentiation of the successive responses at any one test interval. In one example, when the first EPSP was potentiated by about 60 % relative to the control, the relative potentiations declined progressively for the subsequent responses, being only 20 % with the last (cf. Eccles, 1957, Fig. 7*A*, *B*). An even more rapid decline occurred when a repetitive stimulus tested the post-tetanic potentiation at the curarized mammalian neuromuscular junction (Liley & North, 1953). A very rapid decline during a repetitive test was also found by Ström (1951) during post-tetanic potentiation of a monosynaptic reflex.

These results may be taken to indicate that large reserves of available transmitter are not mobilized during post-tetanic potentiation.

Late depression. It was reported and illustrated without comment (Eccles *et al.* 1959, Figs. 3, 6) that some minutes after the conditioning tetanus, the post-tetanic potentiation occasionally passed over to a depression that persisted for many minutes. A comparable depression of the EPSP occurred in Fig. 8*B* from 4 to 7 min post-tetanically, and it had passed off by 10 min. Since the sizes of the control EPSP's had not varied by more than 2% for a period of 30 min, including the 10 min period of Fig. 8*B*, it can be presumed that there was a genuine depression of as much as 7% in synaptic efficacy and not some transient defect in the intracellular recording. Such transient defects frequently arise when movement causes the micro-electrode to be no longer sealed effectively in the motoneuronal membrane. As would be expected, all excitatory synaptic potentials are then similarly diminished, and there is a simultaneous fall in the membrane potential. Such defects of intracellular recording can be detected and allowed for if a synergic excitatory path is employed as a control test throughout the whole of the post-tetanic period.

In each sweep of the inset records of Fig. 9*A* two EPSP's were set up in a gastrocnemius motoneurone, the first by a volley from the lateral gastrocnemius-soleus (LGS) nerve, the second by a medial gastrocnemius (MG) volley. Both before and after the conditioning tetanization of the MG nerve (400/sec for 10 sec at the arrow) the monosynaptic EPSP's were evoked every 2 sec and three traces were superimposed on each record. As would be expected, the large increase in the MG EPSP at 4 sec post-tetanically contrasted with the absence of any appreciable change in the LGS EPSP, which remained virtually constant throughout the whole post-tetanic test period. Each of the points plotted for the post-tetanic period in Fig. 9*A* was obtained by expressing the ratio of the MG EPSP to the LGS EPSP at that time relative to the ratio obtaining before the conditioning tetanus. Since the effects of any transient changes in the intracellular recording were thus eliminated, the prolonged post-tetanic depression of EPSP in Fig. 9*A* (cf. the inset records at 154 and 220 sec) must be due to a diminution of synaptic efficacy. Usually the post-tetanic depression of the EPSP was much less (cf. Fig. 9*B*) and sometimes it was not detectable. The series of Fig. 7*A–D* suggests that post-tetanic depression may be submerged beneath the prolongation of the post-tetanic potentiation that occurs after a long conditioning tetanus. Possibly the degree of submergence beneath the potentiation accounts for the variations in the prominence of the depression between different series both on the same and on different motoneurones.

IPSP potentiation. The IPSP (inhibitory post-synaptic potential) pro-

duced by 1a impulses is also often potentiated after a conditioning tetanus (R. M. Eccles & Lundberg, 1958), corresponding to the post-tetanic potentiation of direct inhibition (Lloyd, 1949; Wilson, 1958), but the potentiation was usually much less than for EPSP's of the same motoneurone (Fig. 10). The differences in amount and time course of the potentiation need not indicate that inhibitory synapses differ from excitatory, but may instead be attributable to the interneurone that is interpolated in the inhibitory pathway (cf. R. M. Eccles & Lundberg, 1958).

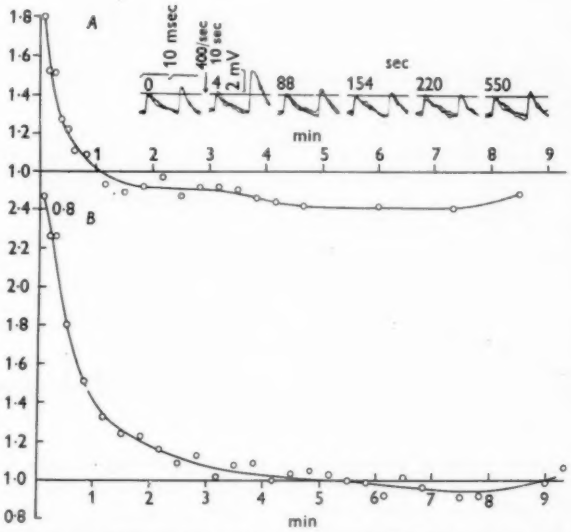

Fig. 9. *A*. Post-tetanic potentiation of monosynaptic EPSP's generated in a gastrocnemius motoneurone (resting potential, − 65 mV) by lateral gastrocnemius and medial gastrocnemius volleys, as shown in the inset records. The conditioning tetanus (400/sec for 10 sec) was applied to the medial gastrocnemius nerve as shown and the subsequent records are at the indicated intervals in seconds after the end of the tetanus. Note post-tetanic depression of the testing EPSP relative to the control at 154 and 220 sec. The size of the MG EPSP is plotted relative to the LG EPSP, as described in the text. *B*. Similar plot for another gastrocnemius motoneurone.

Early potentiation. Brief conditioning tetani at a sufficiently high frequency are followed by a very early and evanescent potentiation, which was originally revealed by increases both of monosynaptic reflexes and of extracellular synaptic potentials (Eccles & Rall, 1951*b*; Lloyd, 1952; Beswick & Evanson, 1955*b*; Wilson, 1958). When studying the changes in synaptic efficacy within 2 sec after brief repetitive stimulations, only one testing stimulus could be employed after each conditioning tetanus, and

the tetani had to be spaced at least 10 sec apart so as to minimize cumulative effects during a prolonged experimental series. As shown in the inset records of Fig. 11*A–C*, the testing intracellular responses were photographed at high speed by expanding the part of the trace on which they occurred. It was thus possible to measure the steepest part of the rising slopes of the EPSP's, and so to determine the potentiation of the EPSP before the slope was increased by the superimposed spike potential. In some experiments the maximum slope was measured as the summit height of electrically differentiated records (cf. the inset records of Figs. 7, 8, 10).

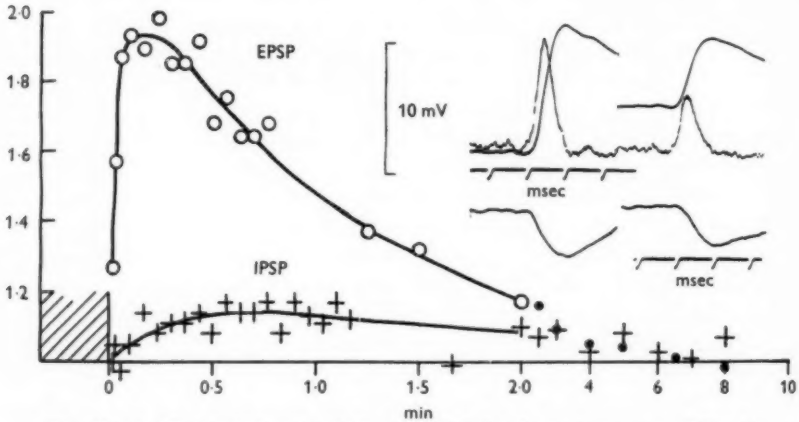

Fig. 10. ○, Post-tetanic potentiation of EPSP for the motoneurone of Fig. 7; +, potentiation of an IPSP generated in the same motoneurone by a quadriceps Group Ia volley. The conditioning tetanus in both cases was 640/sec for 20 sec, the insets showing the EPSP and IPSP at maximum potentiation, and the control. As in Fig. 7, the EPSP has been differentiated.

There were 40 conditioning stimuli for each of the series of Fig. 11*A–C*, but the frequencies were 640/sec, 400/sec and 100/sec for *A*, *B* and *C* respectively. The conditioning tetanus at 400/sec was almost as effective as that at 640/sec, but there was no potentiation whatever after 40 impulses at 100/sec, as also may be seen in the inset records. A conditioning tetanus at 200/sec was not employed in the series of Fig. 11*A–C*, but in other experiments it always gave a small potentiation, whereas 100/sec was always followed by a depression. For example, the curves of Fig. 11*D* give the time courses of the conditioning produced by 40 impulses over a wide range of frequencies in another experiment. Post-tetanic depression was regularly observed after slow conditioning frequencies. Thus the curves of Fig. 11*E* show that there was actually an increasing depression after 57/sec for 0·2 sec whereas after higher frequencies for the same duration

there was considerable potentiation at 200/sec and virtually no change at 100/sec. In this series the size of the last EPSP of the conditioning series was plotted (the initial points on the curves), it being assumed that this would be approximately the size of a test response at the stimulus interval after the end of the conditioning tetanus. There was thus an increasing depression for some time after the slow conditioning tetanus; but after 100/sec there was a potentiation above the level obtaining during the conditioning tetanus.

These potentiations of the EPSP after brief conditioning tetani correspond closely with the observations on reflex potentiation under comparable conditions (Eccles & Rall, 1951*b*; Lloyd, 1952; Beswick & Evanson, 1955*b*). For example, after a high-frequency tetanus of 40–100 volleys

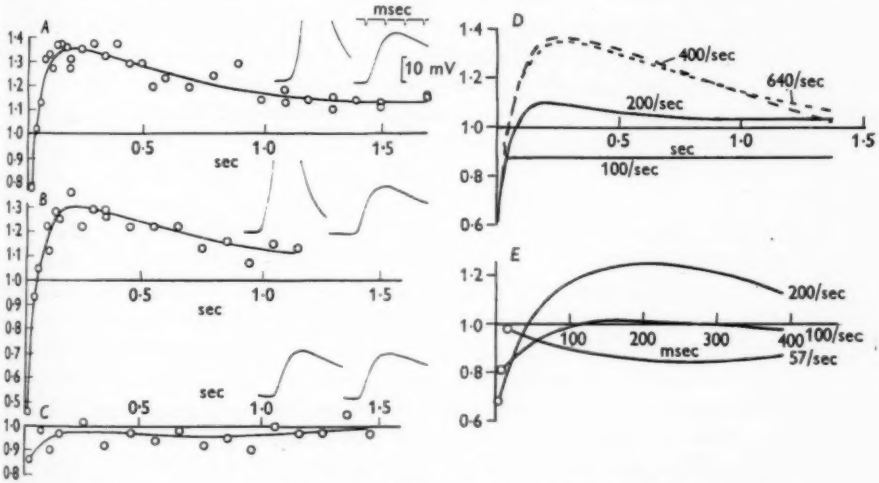

Fig. 11. Early post-tetanic potentiation and depression following brief conditioning tetani. In *A–C* the points plot the potentiation of the steepest part of the rising slope of the EPSP's as a fraction of the mean control; intracellular records from the biceps-semitendinosus motoneurone (resting potential, −60 mV) are shown as insets, the first being the response at about 0·2 sec post-tetanically, the second the control; note spike origin in *A* and *B*. Conditioning tetani of 40 impulses, the respective frequencies being 640/sec, 400/sec and 100/sec for *A*, *B* and *C*. *D* gives curves plotted on the same co-ordinates for a series such as *A–C* in another biceps-semitendinosus motoneurone (resting potential, −67 mV). The conditioning tetani are again 40 impulses. Virtually the same potentiation is produced by conditioning at 640 and 400/sec, 200/sec is intermediate, and 100/sec purely depressant. *E* gives curves on same co-ordinates for the post-tetanic effect of brief tetani at different frequencies for the same duration (0·2 sec). The initial points mark the size of the last EPSP of the conditioning tetanus. Note potentiation after 200/sec, depression after 57/sec with an intermediate response after 100/sec.

the potentiation of monosynaptic reflexes began at about 50 msec, reached a maximum at 200 msec, and thereafter rapidly declined for 1–2 sec.

It is of particular significance that the frequency of the conditioning tetanus plays such a decisive part in setting the level of post-tetanic potentiation (cf. Beswick & Evanson, 1955*b*). Since the total duration of the conditioning tetanus was only 0·4 sec in Fig. 11*C*, and since the potentiations in *A* and *B* had declined very little by 0·4 sec after the tetanus, it is evident that the increased temporal spread of the conditioning volleys was not responsible for the absence of potentiation in Fig. 11*C*. It is the frequency rather than the number of impulses that is of particular significance in producing post-tetanic potentiation. However, to some extent number of impulses can compensate for low frequency; for example in Fig. 8*D*, 3200 impulses at 100/sec gave a potentiation of 1·25 which ran a very prolonged time course, whereas after 40 impulses at 100/sec the potentiation was inadequate to compensate for the depression (Fig. 11*C*, *E*). Similarly Lloyd (1949) reported that post-tetanic potentiation of monosynaptic reflexes always occurred after prolonged repetitive stimulation at a frequency as low as 50/sec.

DISCUSSION

The present investigation has been concerned solely with the potentials generated in motoneurones by afferent impulses successively activating the same synapses, and has been almost entirely restricted to monosynaptic excitatory action. According to present concepts, the various potentiated and depressed states of synaptic action could occur in four ways: (i) block (or relief of block) of impulse transmission at sites of low safety factor in the presynaptic pathways, particularly at branching points (cf. Krnjević & Miledi, 1958); (ii) increase in size of the presynaptic impulse during the after-hyperpolarization that follows repetitive activity (Lloyd, 1949, 1952; Eccles & Rall, 1951*b*; Liley & North, 1953; Wall & Johnson, 1958; Eccles & Krnjević, 1959*a*, *b*); (iii) change in the availability of transmitter substance in the presynaptic terminals, more or less being liberated by a given size of presynaptic impulse (Brown & Feldberg, 1936; Perry, 1953; Emmelin & Macintosh, 1956; Liley & North, 1953; Liley, 1956*a*, *b*; Eccles, 1957; Hubbard, 1959); (iv) depression of the effectiveness with which the transmitter causes post-synaptic depolarization, an effect which has been called receptor desensitization (Thesleff, 1955; Katz & Thesleff, 1957; Axelsson & Thesleff, 1958). It should be noted in this context that during post-tetanic potentiation there is no appreciable increase in the sensitivity of the post-synaptic membrane to the transmitter substance (Larrabee & Bronk, 1947; Hutter, 1952). Likewise, during post-tetanic depression, testing of the motoneurones by a converging excitatory path

showed that there was no change in the excitability (Beswick & Evanson, 1957).

In many respects the end-plate potentials generated at the mammalian neuromuscular junction during and after repetitive stimulation (Lundberg & Quilisch, 1953; Liley & North, 1953; Liley, 1956*a*, *b*) exhibit phases of potentiation and depression resembling those here described for the potentials generated at central synapses. In addition, after repetitive stimulation the frequency of miniature end-plate potentials parallels the potentiation of the neurally evoked end-plate potentials (Liley, 1956*a*, *b*; Hubbard, 1959). The frequency of miniature end-plate potentials can be regarded as a measure of the availability of transmitter; hence this parallelism provides strong support for factor (iii) above. In the first instance it will therefore be expedient to attempt to explain the experimental observations during and after brief tetani by postulated changes in the availability of transmitter (cf. Liley & North, 1953). This procedure is also chosen because factors (i), (ii) and (iv) probably achieve significance only after prolonged stimulation.

In the simplest experiment it has been shown that, after a single conditioning impulse, a brief phase (up to 200 msec) of potentiation is superimposed on a depression of more than 1 sec duration (Figs. 1, 2). An important correlation is that at the mammalian neuromuscular junction the frequency of miniature end-plate potentials is increased for as long as 200 msec after a single impulse (Liley, 1956*a*; Hubbard, 1959). Both the potentiation and depression are cumulative during repetitive stimulation so that at frequencies above 10/sec a steady state of synaptic efficacy is established in about 0·2 sec. At low frequencies (1–10/sec) depression is dominant, but with the higher frequencies there is increased summation of the briefer potentiating phases (Figs. 2, 4, 5) and at frequencies of 40–100/sec the potentiation may even dominate the depression (Figs. 2*B*, 3*B*). With still higher frequencies the individual synaptic actions are depressed, the approximate inverse relationship to frequency indicating that the actual rate of liberation of transmitter reaches a maximum at frequencies in excess of 250/sec (Fig. 6*B*).

It seems possible to correlate these changes in synaptic efficacy during stimulation with the changes observed within 1–2 sec after these brief tetani (Fig. 11). On termination of repetitive synaptic stimulation at slow frequencies (100/sec or less), there is a subsequent prolonged period (seconds) of depression of the testing EPSP. This depression is greatest after frequencies that are optimal for the depression during repetitive stimulation, i.e. for frequencies of 5–20/sec (cf. Figs. 3, 4, 5). Evidently this is the same depression which was observed after single conditioning EPSP's (Figs. 1, 2) and which occurs during slow frequencies of stimulation.

It is postulated that EPSP's are depressed under all these conditions because there is a preponderant effect of depletion of the available transmitter. At higher frequencies mobilization of the available transmitter appears to become progressively more dominant, so that at about 100/sec there is an approximate balance between potentiation and depression for a second or so after the brief conditioning tetani (cf. Fig. 11*C*, *D*, *E*). After still higher frequencies potentiation is dominant, there being virtually the same high level of potentiation after brief tetani in the frequency range of 300 to 800/sec (Figs. 11*A*, *B*, *D*). This range corresponds approximately to the range for optimum rate of liberation of transmitter during repetitive stimulation.

On termination of repetitive synaptic stimulation at 250/sec or higher, there is a sudden cessation of the liberation of transmitter, which had been running at the maximum attainable rate; albeit with an output per impulse much below the level for a single impulse under resting conditions. It would therefore be expected that, after cessation of this maximum drain of transmitter from the synaptic knob, there would be an accumulation of available transmitter therein above the resting level; as a consequence a testing impulse would cause an increased liberation of transmitter, so giving the potentiated EPSP seen in Fig. 11. The time course of this early post-tetanic potentiation indicates that available transmitter goes on accumulating for as long as 200 msec after repetitive synaptic stimulation has ceased and then slowly declines over several seconds. During lower frequencies of synaptic stimulation (150–200/sec) there is a lower rate of liberation of transmitter (Fig. 6*B*), and correspondingly there is a lower potentiation after cessation (Fig. 11*D*).

Thus in general the EPSP's generated during and after brief repetitive stimulation can be satisfactorily accounted for by the postulate that each presynaptic impulse exerts two opposing actions on the transmitter mechanism, a depletion of available transmitter and a mobilization which may be more or less than compensatory. At any frequency of presynaptic activation a balance is soon reached between these two effects, so giving the steady state (Fig. 3). It seems furthermore necessary to postulate that with impulses in quick succession (40/sec or more), there is some serial facilitatory action causing a more effective mobilization of available transmitter and a consequent potentiated output of transmitter due to the mobilization process dominating the depletion.

In preliminary observations Liley (1956*b*) showed that after as few as 100 impulses (200/sec for 0·5 sec) there was a considerable increase in frequency of miniature end-plate potentials at the mammalian neuromuscular junction, the relative increase being larger than the potentiation of the end-plate potential. Systematic investigations with brief conditioning tetani of various durations and frequencies have shown that the

post-tetanic increase in frequency of miniature potentials was always more than the potentiation of the end-plate potential; hence it can be concluded that the post-tetanic increase in available transmitter is sufficient to explain both the size and duration of the potentiation (Hubbard, 1959).

It has been observed that after cessation of a prolonged (10 sec or longer) high-frequency tetanization of synapses, there was a phase of depressed synaptic transmission for as long as 1 sec, maximum post-tetanic potentiation occurring as late as 30 sec after the tetanus (Fig. 7*D*; Lloyd, 1949, 1952; Eccles & Rall, 1951*b*; Ström, 1951; Eccles *et al.* 1959). This delayed attainment of maximum potentiation also occurred after prolonged tetanization of sympathetic ganglia (Larrabee & Bronk, 1947) and of the curarized mammalian neuromuscular junction (Liley & North, 1953; Liley, 1956*a*; Hubbard, 1959). In the latter situation the frequency of miniature end-plate potentials was usually maximal immediately after the tetanus, indicating that available transmitter was then at a maximum, yet there was not a corresponding release by the testing impulse. Probably this discrepancy is attributable to failure of the presynaptic impulse to invade all the synaptic terminals, an explanation (cf. factor (i), p. 390) which also seems likely for the delayed summit of post-tetanic potentiation of central synaptic transmission.

Two explanations (factors (ii) and (iii) above) are available for the prolonged potentiation that follows a long high-frequency tetanus (cf. Figs. 4, 5). It has now been established that after a tetanus there is a large and prolonged after-hyperpolarization of the presynaptic fibres, with, as a consequence, an increase in the presynaptic spike potential (Lloyd, 1949; Eccles & Rall, 1951*b*; Wall & Johnson, 1958; Eccles & Krnjević, 1959*a*, *b*). Such an increased presynaptic spike can be expected to have an increased synaptic excitatory action (cf. Lloyd, 1949; Eccles & Rall, 1951*b*), and it is now known that under such conditions there is an increased emission of quanta of transmitter (del Castillo & Katz, 1954*c*; Liley, 1956*b*). A particularly large increase in synaptic action was observed in the stellate ganglion of *Loligo* when the presynaptic spike was increased as a consequence of an applied anelectrotonus (Hagiwara & Tasaki, 1958). Thus there is an impressive array of evidence indicating that prolonged post-tetanic potentiation arises on account of the increased size of the presynaptic impulse (factor (ii) above).

Yet there is no less strong evidence in support of an explanation by the increased availability of transmitter (factor (iii) above). If it be assumed that the frequency of miniature end-plate potentials is a measure of availability of transmitter, there is a close correlation between the magnitude and time course of the prolonged post-tetanic potentiation and the increase in available transmitter, the only significant discrepancy being the delayed

rise of the former (Liley, 1956*a*, *b*; Brooks, 1956; Hubbard, 1959). Further evidence of a negative character also supports an explanation by factor (iii). In many cases (Liley & North, 1953; Eccles & Rall, 1951*b*) potentiation of the presynaptic spike potential runs a briefer time course than the synaptic transmission. Sometimes, however, the agreement may be very close (Lloyd, 1949; Wall & Johnson, 1958).

In conclusion, it may be accepted that post-tetanic potentiation after a prolonged conditioning tetanus is attributable both to potentiation of the presynaptic impulse and to increased availability of transmitter. It has been suggested above that the latter explanation accounts satisfactorily for the early post-tetanic potentiation after a brief conditioning tetanus (about 20–200 stimuli at high frequency); and presumably, as the conditioning tetanus is prolonged, after-hyperpolarization with a consequent potentiation of the presynaptic spike becomes an increasingly important factor. Possibly the depression and eventual abolition of the early potentiation that occurs when the conditioning tetanus is increased beyond a few hundred impulses (Eccles & Rall, 1951*b*; Lloyd, 1952) are due to the presynaptic after-hyperpolarization causing an initial post-tetanic phase of partial presynaptic block.

Brief reference should be made to the small and inconstant phase of late depression that follows the potentiation after a long conditioning tetanus (cf. Fig. 9; Eccles *et al.* 1959). Much larger post-tetanic depressions of this type have been found with synaptic transmission through the lateral geniculate ganglion (Evarts & Hughes, 1957; Hughes, 1958; P. O. Bishop & W. Burke, unpublished observations). As a preliminary suggestion it seems likely that the receptor desensitization found at the neuromuscular junction (Thesleff, 1955; Katz & Thesleff, 1957; Axelsson & Thesleff, 1958) provides the most likely explanation (factor (iv) above). The prolonged synaptic stimulation can be envisaged as causing a blockage of many receptor sites on the post-synaptic membrane, just as occurs during prolonged treatment of the neuromuscular junction with a low concentration of acetylcholine. Such a depression would not be manifest until after the decline of the potentiation due to increase in available transmitter.

The principal interest in the present investigation arises in relation to the mechanism ensuring effective synaptic action at high frequency of activation. Previous investigations have given rise to the suggestion that repetitive synaptic activation involves two opposing processes, depletion of the available transmitter and a compensatory mobilization of transmitter (Feng, 1941; Larrabee & Bronk, 1947; Hutter, 1952; Lundberg & Quilisch, 1953; Liley & North, 1953; Eccles, 1953, 1957; del Castillo & Katz, 1954*b*; Beswick & Evanson, 1955*b*; Liley, 1956*b*). Del Castillo & Katz (1954*a*) made the important observation that a presynaptic impulse

induced potentiation even when no transmitter liberation occurred; thus depletion of available transmitter is not a prerequisite for a reaction giving an excess. Since the increase in available transmitter often overcompensates for any depletion that arises during repetitive stimulation (Figs. 2*B*, 3*B*; del Castillo & Katz, 1954*b*), depletion is also contra-indicated as a cause of the mobilization. Apparently the presynaptic impulse initiates the process of transmitter mobilization, and there is a large facilitation of this process when presynaptic impulses follow at high frequency. In this way high frequency of synaptic activation calls forth a mobilization of transmitter that ensures a greater synaptic effectiveness for rates of synaptic activation up to 250/sec (cf. Fig. 6).

If, as seems likely, the quantal liberation of transmitter is due to the bursting of synaptic vesicles into the synaptic cleft (del Castillo & Katz, 1956; Katz, 1958), the available transmitter at any one instant would be the transmitter in vesicles in immediate juxtaposition to the presynaptic membrane, for only such vesicles could be ejected by an impulse. There may be very few vesicles in such a strategic position; consequently depletion may be evident even after only one impulse (cf. Figs. 1, 2). Besides causing the ejection of some vesicles the presynaptic impulse can be envisaged as causing other vesicles to move into the strategic zone. This movement would be greatly potentiated by impulses in quick succession, but no such interaction occurs with longer intervals (30 msec or longer) between successive presynaptic impulses; hence depletion then dominates mobilization (cf. Figs. 4, 5). Electron-microscopic investigations may eventually provide a crucial test for these speculations on the structural correlations of the depletion and mobilization phenomena of repetitively activated synapses.

SUMMARY

1. Repetitive synaptic stimulation has been studied in detail by intracellular recording of the excitatory post-synaptic potentials (EPSP's) of motoneurones activated monosynaptically, it being assumed that these synaptic potentials give a measure of the size of the transmitter action.

2. A single synaptic activation is followed by two opposed processes; a brief phase of enhanced action (200 msec) is superimposed and usually submerged by a depression lasting for several seconds.

3. During repetitive stimulation (10–600/sec) a steady state is attained in about 200 msec. There is usually depression below the initial size, but with some EPSP's there may be potentiation.

4. There is increasing depression of the EPSP's as the frequency of activation is raised above 0·3/sec, and a minimum of 70–85 % occurs in the range 4–20/sec. At higher frequencies there is always a relative potentiation, a maximum of 77–128 % being attained at 30–100/sec. There is a

good correlation between responses to two volleys on the one hand and the steady state during repetitive stimulation on the other.

5. At higher frequencies of stimulation there is a progressive decline of the individual EPSP's, and above 250/sec the size of the EPSP is directly proportional to the stimulus interval, indicating the attainment of a maximum rate of transmitter output.

6. The changes in synaptic efficacy following repetitive stimulations of a wide range of frequencies and durations have been measured by the sizes of the EPSP's evoked by a testing volley at various times thereafter.

7. Brief tetani of 200/sec or higher are followed by an early potentiation, just as with monosynaptic reflexes.

8. After long conditioning tetani there was also a close parallel between potentiation of EPSP's and reflexes, the potentiation being low with frequencies below 300/sec. A later post-tetanic depression of EPSP's is also described.

9. There is a general discussion of the relationship of these observations to the mode of operation of synaptic knobs under various frequencies of activation. Post-tetanic potentiation is discussed in relation to the alternative explanations, transmitter mobilization in synaptic knobs, and after-hyperpolarization giving larger presynaptic spikes.

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DIFFERENTIATION OF FAST AND SLOW MUSCLES IN THE CAT HIND LIMB

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Ranvier (1874) described the first systematic investigation of the contractions of the slow and fast limb muscles, and a further systematic investigation was made by Fischer (1908). Banu (1922), Denny-Brown (1929*b*) and Koschtojanz & Rjabinowskaja (1935) showed that all limb muscles were slow at birth and differentiation into the fast and slow types occurred in mammals during the first few weeks after birth. Recently it has been found that motoneurones innervating slow muscles have as a rule much longer after-hyperpolarizations following the discharge of an impulse than do the motoneurones supplying fast muscles (Eccles, Eccles & Lundberg, 1958). Thus the characteristic frequencies of discharge of these two types of motoneurones (Denny-Brown, 1929*a*; Granit, Henatsch & Steg, 1956; Granit, Phillips, Skoglund & Steg, 1957) are explicable by their intrinsic properties. The shorter after-hyperpolarization of motoneurones supplying fast muscles allows their fast frequency of firing, which is appropriately related to the contraction time of their muscles; and complementarily those motoneurones with longer after-hyperpolarizations have as a consequence frequencies of discharge appropriate to the slow muscles they innervate. It was, therefore, of interest to re-investigate the process of differentiation into fast and slow muscles, in an attempt to determine whether this appropriate matching of motoneurones to muscles was brought about by motoneurones influencing muscle differentiation, or, vice versa, by muscle influencing motoneurones.

The present paper describes a detailed study of muscle differentiation as defined by various measurements of the speed of muscle contraction. It also describes the effects of various nerve lesions on this differentiation. A more analytical investigation is described in the following paper (Buller, Eccles & Eccles, 1960).

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METHODS

The animals (kittens and cats) were anaesthetized with pentobarbital sodium. The muscles under investigation were isolated by dissection, care being taken to preserve their blood supply. The motor nerves were cut centrally and mounted on stimulating electrodes. Full precautions have been taken to ensure that the muscle contractions approximated as closely as possible to isometric contractions: for the leg muscles steel drills transfixed the distal end of the femur and the distal ends of the tibia and fibula; for the thigh muscles the drills were inserted through the greater trochanter and the distal end of the femur. The drills were clamped in chucks which in turn were fixed to a massive steel frame to which the recording strain gauge (Statham Gl–8–350 or Gl–80–35) was also fixed by an assembly giving great flexibility of adjustment. The muscle tendon (or the patella in the case of crureus) was tied firmly to a short steel hook directly linked to the strain gauge; care was taken to align the strain gauge with the direction of muscle pull, which of course was kept as close as possible to its natural line of pull. The direction of muscle pull was about 60° from the vertical so that it was possible to have the muscle immersed in a paraffin pool with its motor nerve readily accessible. Controlled heating and stirring of the pool ensured that its temperature was almost always in the range 37–38° C. This temperature control was important because the speed of muscle contraction has a fairly high temperature coefficient; $T_{10} = 1{\cdot}53$ according to Gordon & Phillips (1953). Despite the oblique angle of the muscle pull there was a negligible sagging of the muscle, because its weight was greatly reduced by its immersion in paraffin and it had a fairly high initial tension—usually more than ten times the weight of the muscle.

Considerable effort has been made to ensure that the various muscles were contracting under comparable conditions of initial tension. The relationship of initial tension to duration of the isometric twitch was described in detail by Fulton (1925, 1926), but unfortunately the twitches were distorted by friction of the myograph bearing, as revealed by the angle between the summit and the falling phase (Cooper & Eccles, 1929). A re-investigation was therefore necessary, and Fig. 1 shows the effect of length on the initial tension and on the time course and size of the twitch. Measurements from the series partly shown in Fig. 1*A–E* are plotted in Fig. 1*F* with the muscle lengths as abscissae; and as ordinates the initial tensions, the twitch tensions, the twitch contraction times (onset to summit), and the half-decay times (summit to half-decay). It will be seen that around the optimum condition for active tension development (cf. Banus & Zetlin, 1938) progressive lengthening of the muscle causes lengthening both of the contraction time of the twitch and of the time from summit to half-relaxation. It was therefore necessary to adopt some criterion in order that the contractions of different muscles could be compared under standard conditions. The standard adopted was the muscle length giving the maximum twitch tension, as indicated by the vertical line in Fig. 1*F*. In all figures zero on the tension scales has indicated zero muscle tension.

The muscle contractions have been evoked by stimulating the appropriate muscle nerve with brief electrical pulses about 0·1 msec in duration and about twice maximum strength. Several twitches were recorded at each length of the muscle, but measurements were made only of the twitches at the optimum length and all the values plotted are the means of these measurements. A decade-counter device was constructed so that the duration of the twitch contraction time could be read off on the lower beam of the oscilloscope (cf. Figs. 2, 11) at the same time as the upper beam was recording the time course of the twitch (Buller, unpublished). The tetani were recorded only at the initial tension giving optimum twitches, and were evoked by brief repetitive electrical pulses, as for the twitches. Usually the series was from the slowest to the fastest with a final return to the slowest frequency; the frequencies were standardized at 13, 19, 32, 46, 66, 104 a second. There was an interval of several seconds between successive tetani, but not long enough to allow for dissipation of the

post-tetanic potentiation of the muscle twitches (Brown & von Euler, 1938; Feng, Lee, Meng & Wang, 1938; von Euler & Swank, 1940). Post-tetanic potentiation was, however, much smaller for tetani, so no large error was introduced by calculating the tetanus-to-twitch ratios relative to the twitch height at the onset of the tetanic investigations. The durations of the tetani were kept very short (about 0·2 sec) so as to minimize post-tetanic potentiation.

In separating lateral from medial gastrocnemius it is necessary to divide a small slip of muscle that arises from a common tendon. Separation between lateral gastrocnemius and plantaris is not possible by dissection because both muscles have a large origin from a common tendon. No appreciable error has been introduced thereby, because these muscles normally respond with twitches having about the same time courses and they have not been employed in the more analytic procedures involving nerve cross-unions, as described in the subsequent paper (Buller *et al.* 1960). The crureus muscle can be separated from rectus femoris and vastus lateralis, but separation from vastus medialis is not possible. Contractions

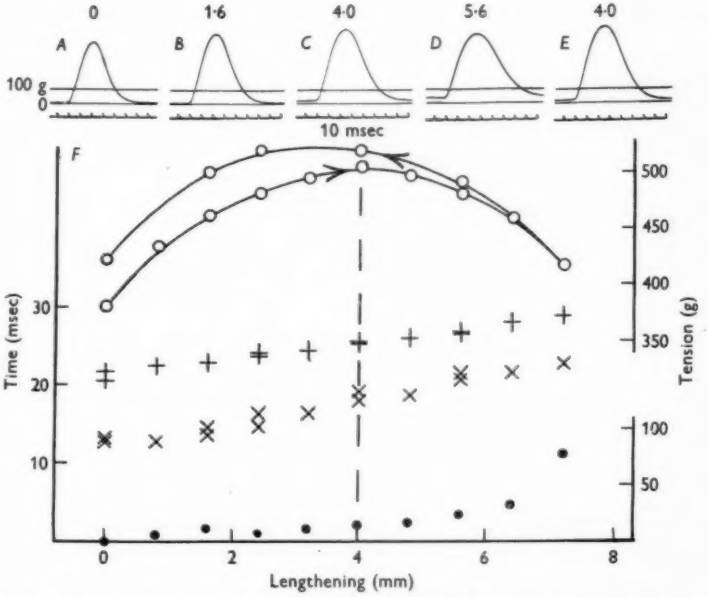

Fig. 1. Isometric twitch contractions of flexor digitorum longus muscle (FDL) of a spinal kitten 13 weeks old. Progressive lengthening from an arbitrary zero length in *A*, the successive lengthenings being 1·6 mm for *B*, 4·0 mm for *C* and 5·6 mm for *D*, with a return in *E* to the same length as *C*. Note tension scale of 100 g. Weight of kitten = 1·5 kg. In *F* the whole series partly shown in Fig. 1 *A–E* is plotted with the lengthenings as abscissae; and as ordinates, the initial tension (●), the twitch tensions (○), the contraction times, i.e. times from onset to summit of the twitch (+) and the half-relaxation times, i.e. times from summit to half-relaxation (×). Throughout this paper the various measurements on the muscle twitches have been made under the conditions giving the greatest twitch contraction, i.e. at the vertical line in Fig. 1 *F*. Twitch tensions were rather higher on the return from the greatest lengthening, but muscles were not ordinarily subjected to such excessive stresses. Note break in tension scales (right-hand ordinate).

of crureus independently of vastus medialis were secured by cutting all nerve branches to vastus medialis. As a check on the effectiveness of this separation of the slow muscle, crureus, from the fast muscle, vastus medialis, the whole of the branch from quadriceps nerve to crureus and vastus medialis was often cut after the contractions of crureus had been recorded, leaving intact only the crureus nerve that arises from the nerve to vastus lateralis. Invariably it was found that this fraction of crureus gave twitches and tetani closely comparable to those of the whole crureus. All other muscles were readily isolated.

RESULTS

Normal differentiation of fast and slow muscles

Muscle twitches in a representative series of muscles are shown in Fig. 2 for animals ranging from 20 hr after birth to 28 weeks. In confirmation of Banu (1922) and Denny-Brown (1929*b*), all muscles are equally slow in the

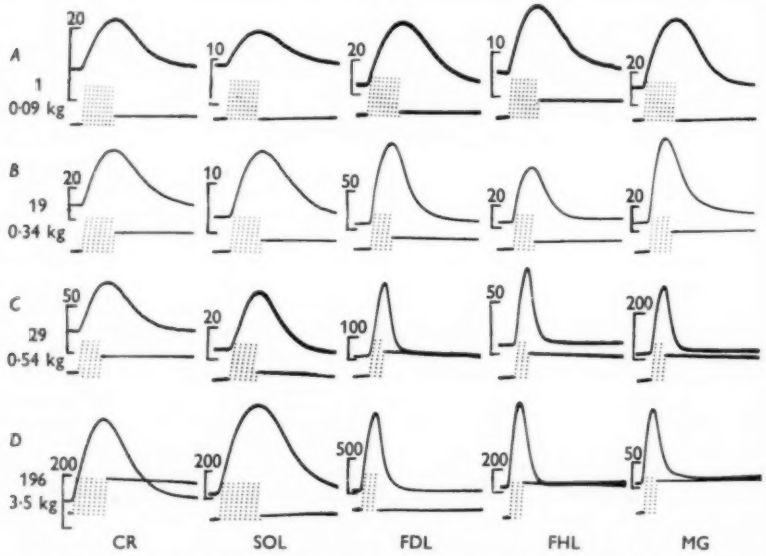

Fig. 2. *A–D.* Isometric twitch contractions of five different leg muscles in the cat at the ages in days and weights shown, and with contraction times indicated in milliseconds by the decade-counter device. CR = crureus; SOL = soleus; FDL = flexor digitorum longus; FHL = flexor hallucis longus; MG = medial gastrocnemius. Tension scales indicated in grams, zero giving zero tension on muscle.

new-born kitten (*A*), with a contraction time of about 80 msec and a half-relaxation time also of about 80 msec. Within a few days the fast muscles are clearly differentiated from the slow muscles, soleus and crureus (Fig. 2*B*), and by about four weeks (Fig. 2*C*) the fast muscles have attained a speed which is virtually unaltered thereafter, as is indicated by Fig. 2*D* at

28 weeks. It appears from Fig. 2*C* that in the 4-week kitten the slow muscles are appreciably faster than in the 28-week kitten, and this effect is well displayed in the plotted series of Figs. 3*D* and 4*D*, both for contraction times and half-relaxation times. The points for the two slow muscles are seen to lie about curves of shortening contraction and relaxation times until minima of just over 50 msec for both are attained at about 5 weeks, but thereafter the twitches become slower and after several weeks reach the adult values of about 70 and 75 msec respectively. On the whole the

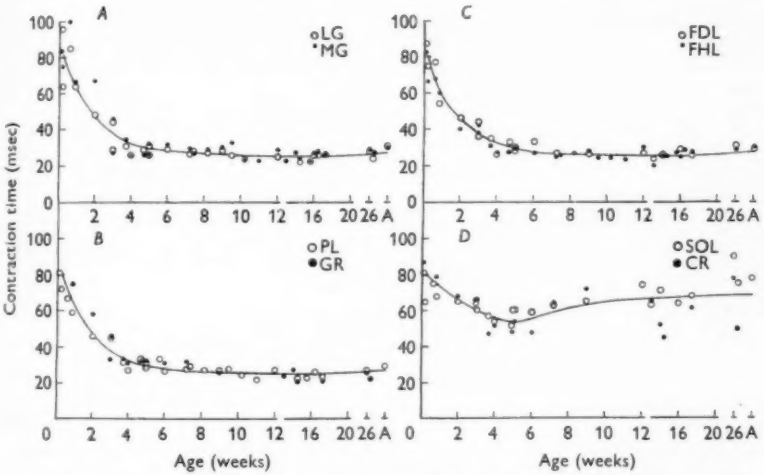

Fig. 3. Contraction times of the various muscles as ordinates against ages of cats as abscissae for six fast muscles, as indicated by the symbols in *A*, *B*, *C* and for two slow muscles in *D*. Muscles additional to those of Fig. 2 are lateral gastrocnemius, LG, plantaris, PL, and gracilis, GR in *B*. The curves in *A*, *B* and *C* are the same curve that is drawn for the pooled results of these three graphs for the six fast muscles. Note change in age scale beyond 12 weeks, and the final *A* values for adult cats. Usually each of the plotted points represents the mean for the muscles on the two sides. The points at 26 weeks, 26+ weeks, and adults represent the means for several animals, usually three.

crureus tends to be a little faster than soleus (cf. Gordon & Phillips, 1953). In Figs. 3*A–C* and 4*A–C* the six fast muscles are seen to have comparable time courses of differentiation, and the curves drawn in Figs. 3*A–C* and 4*A–C* are in fact the same mean curves derived respectively from the pooled result of the six muscles. The standard curve of fast-muscle differentiation reaches a minimum value of about 25 msec contraction time and 16 msec half-relaxation time at 8–16 weeks, but thereafter there is probably a slight lengthening to the adult values of about 27 and 18 msec respectively.

In Fig. 5*C*, *D*, *E* it is seen that already in the 3-week-old kitten the responses of the fast muscles to repetitive stimulation have approximated to the adult pattern. There is relatively little summation at 13/sec, and with a frequency as high as 46/sec the rhythm of stimulation can be detected on the tetanic plateau. In contrast, the rhythm of stimulation has virtually disappeared at 32/sec with the two slow muscles, crureus and soleus (*A*, *B*). When comparing tetani at various frequencies of fast and slow muscles, it will be seen that comparable degrees of fusion occur when the frequencies are appropriately chosen. For example the fusion for fast muscles at 32/sec in Fig. 5*C–E* is approximately the same as at 13/sec for

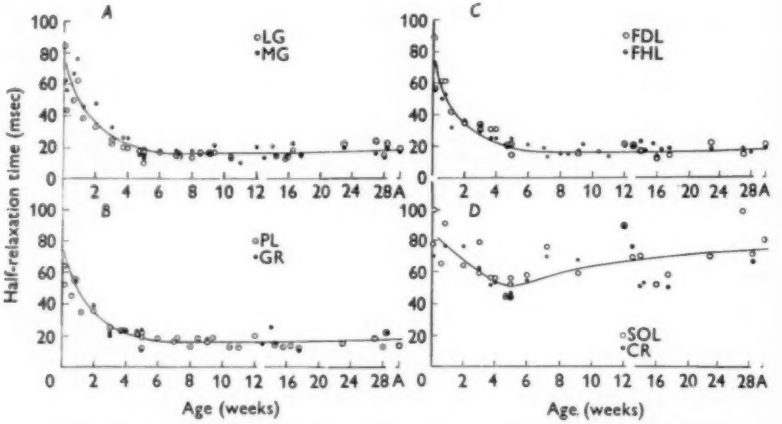

Fig. 4. Half-relaxation times of the various muscles, conventions as for Fig. 3. Note again that the line drawn in *A*, *B* and *C* is for the pooled results of these three graphs for the six fast muscles. Again the age scale is changed beyond 12 weeks.

the slow muscles (*A*, *B*), and again 46 and 66/sec for the fast muscles correspond respectively to 19 and 32/sec for the slow muscles. However, the comparison is not exact because slow muscles have a slowly climbing tetanic plateau, whereas with fast muscles the plateau shows little or no tendency to climb (Fig. 5*C–E* at 66/sec), and may even decline.

When comparing the speeds of different muscles, as exhibited by their responses to various frequencies of stimulation, it has been important to plot the response–frequency curves for these muscles, or, response–stimulus-interval curves as has been done in Fig. 5*F–J*. The frequencies or stimulus intervals are related to the tensions of the summed contractions by S-shaped curves (best seen in *I*, *J*) that attain maxima at frequencies which give a smooth plateau, i.e. at which there is complete fusion of the summed

contraction responses (cf. Adrian & Bronk, 1929; Cooper & Eccles, 1930). A basis for comparison of the speeds of muscles has been conveniently given by the stimulus intervals for the mid points of the S-shaped curves, as shown by the arrows in Fig. 5*F–J*. The characteristic stimulus intervals so determined for the various muscles have been plotted in Fig. 6 against age, so forming another set of curves expressing the time courses of differentiation of muscles (cf. Figs. 3, 4). In the day-old kitten the repetitive responses reveal that all muscles are uniformly slow (cf. Fig. 7), and the adult speed

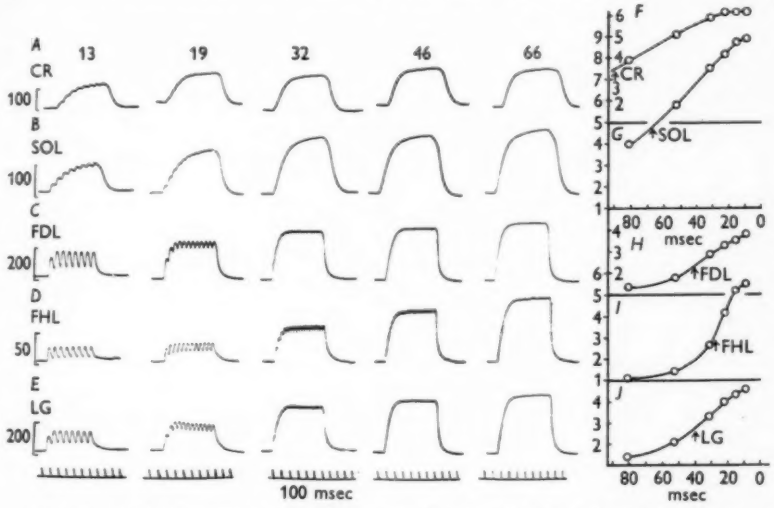

Fig. 5. *A–E*. Responses of five representative muscles of a 3-week kitten, as indicated, to repetitive stimulation at the frequencies per second stated above each column. Note tension scales in grams. At the column on the extreme right (*F–J*) the tetanus:twitch ratios for each muscle are plotted as ordinates against the stimulus intervals of the tetani (i.e. the reciprocals of the frequencies), as abscissae. Note that the longest stimulus intervals are plotted to the left to correspond with the arrangement of the records in each row. The arrows mark on the response–stimulus-interval curves of each muscle the stimulus intervals giving half the maximum tetanic summation, i.e. half way between the twitch tension and the maximum tetanic tension. Weight of kitten, 0·52 kg.

of fast muscles is almost attained by five weeks. The curves of Fig. 6 are less complete than Figs. 3 and 4 because the strain gauge was too weak (maximum only 2·2 kg) to record the tetanic contractions of most fast muscles in kittens of over 16 weeks. A further defect in Fig. 6 is that the durations of the tetani were usually too brief to attain the plateau tensions with the slow muscles, particularly at low frequencies (cf. Fig. 7). As a consequence the plotted points of Fig. 6*D* are often too low, and this may account for the very low values for soleus and crureus at 1–2 weeks.

Despite these defects, Fig. 6 may be regarded as giving a sufficiently reliable index of the differentiation of fast and slow muscles in respect of their repetitive responses, which is seen to follow a time scale approximately comparable with that given by the two previous criteria, contraction times (Fig. 3), and half-relaxation times (Fig. 4).

The unique time courses of differentiation of the speeds of contraction of fast and slow muscles raise the question of the nature of the controlling influences. For example, it can be asked if the influences are inherent in

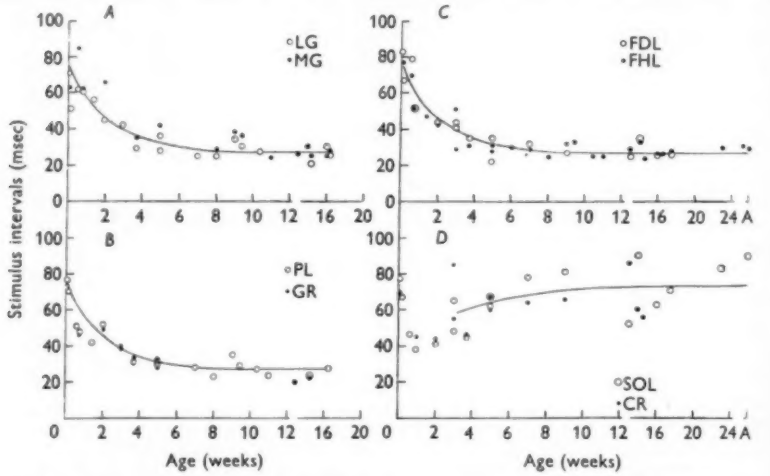

Fig. 6. Stimulus intervals, giving half the maximum tetanic summation, measured as indicated in Fig. 5*F–J*; conventions as for Fig. 3. There is a paucity of values for the older animals because the maximum tetanic tensions were beyond the range of the strain gauge.

the muscles or if they are the result of some action of the nervous system which is different for fast and slow muscles. As a first attempt to decide between these two alternatives, the nervous system has been subjected to various operative procedures in kittens a few days old and the effects of such lesions on muscle differentiation have been examined by later experimental investigations. One kitten could of course be employed only for one such testing period, just as with the normal differentiation curves of Figs. 3, 4, 6.

Effect of spinal cord transection on differentiation of muscle

The spinal cord was transected at the first or second lumbar segment in nine kittens 1–3 days old. A representative series of muscle twitches is illustrated in Fig. 8 for a spinal kitten 13 weeks old. With normal kittens

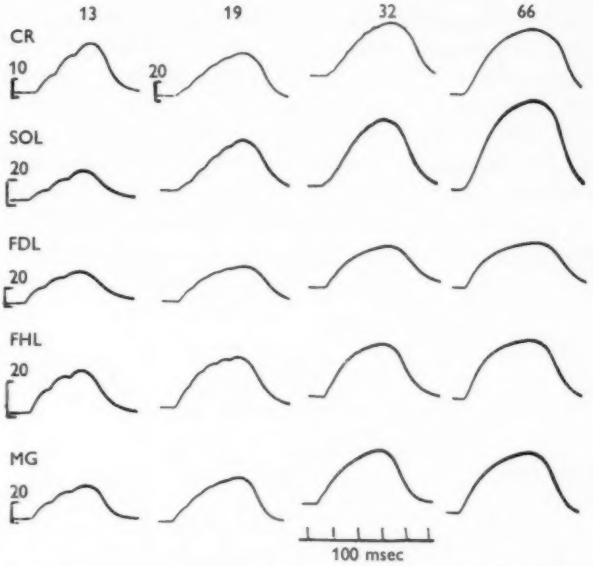

Fig. 7. Tetanic responses of the various muscles of a kitten 20 hr old at the frequencies indicated as in Fig. 5; note that the crureus muscle at the lowest frequency was recorded at higher amplification than the remainder of the series. Tension scales in grams; weight of kitten, 0·09 kg.

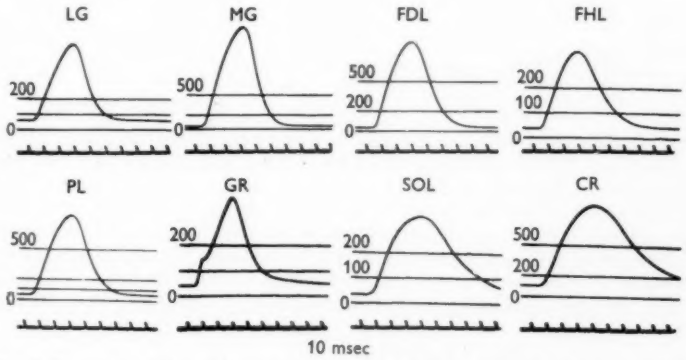

Fig. 8. Twitches of the various muscles indicated in a 13-week-old kitten, spinal cord transected at the L1 level when 36 hr old; note the very fast twitches for crureus and soleus muscles, and the very fast relaxations of the gastrocnemii, and gracilis; the two components of gracilis are very conspicuous. Weight of kitten, 1·75 kg; tension scales in grams, showing as usual the zero tension.

of that age there has been virtually complete differentiation of the slow muscles, the contraction times being in the range 60–90 msec (cf. Fig. 3*D*), and the half-relaxation times 50–100 msec (cf. Fig. 4*D*). In contrast, crureus and soleus in Fig. 8 had contraction times of 33 and 32 msec respectively, and half-relaxation times of 24 and 25 msec respectively. The observations on soleus and crureus contractions in spinal kittens are plotted as open and filled circles in Fig. 9*D* for contraction times, and in Fig. 10*D* for half-relaxation times. Comparison with the standard curves of Figs. 3, 4 (broken lines) reveals that there has been a failure of the differentiation of slow muscles that normally occurs between 5 and 16 weeks; as a consequence soleus and crureus responded by twitches that

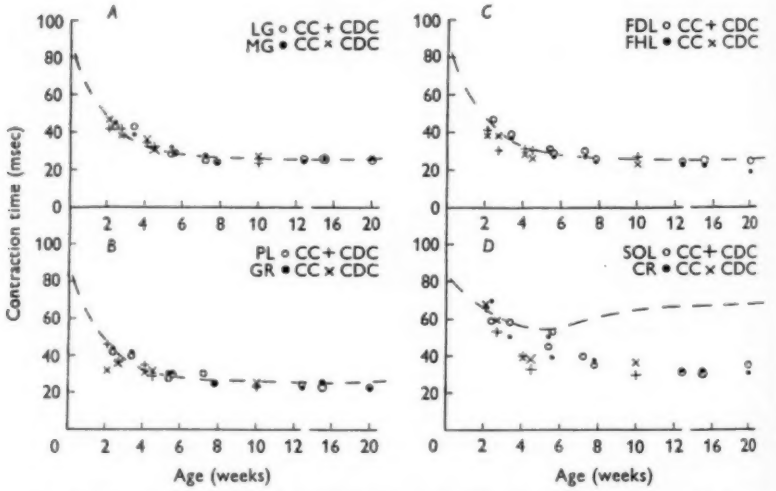

Fig. 9. The open and filled circles show the contraction times for muscle twitches, as in Fig. 3, but for animals in which the spinal cord had been transected when 1–4 days old, as illustrated in Fig. 8 (CC, cord cut; CDC, cord and dorsal roots cut). The crosses show a similar series for muscles as indicated by the symbols, but for animals in which the initial operation gave a complete isolation of the spinal cord from all incoming impulses, as described in the text, a representative series being illustrated in Fig. 11 (CDC = cord and dorsal roots cut). The broken lines show the standard curves for the contraction-time–age curve, derived as in Fig. 3.

were but little slower than the fast muscle responses (cf. Fig. 8). Correspondingly there was a shortening of the stimulus interval for half tetanic summation, the mean value at 10–20 weeks being about 40 msec, which is much closer to the mean value for normal fast muscles, 27 msec, than for normal slow muscles, 73 msec (Fig. 6).

On the other hand the contraction times of the fast muscles of spinal kittens differed very little from the muscles of normal kittens (Fig. 9*A*, *B*, *C*), though possibly the contraction times were a little shortened in the oldest spinal kittens. A much more significant difference appeared in the half-relaxation times of these muscles, particularly the medial and lateral gastrocnemii (Fig. 8), plantaris and gracilis (Fig. 8), the mean values being about 12 msec (Fig. 10*A*, *B*), as against 16–18 msec for normal kittens in the age group of 6–20 weeks (Fig. 4*A*, *B*). As is illustrated in Fig. 8 and

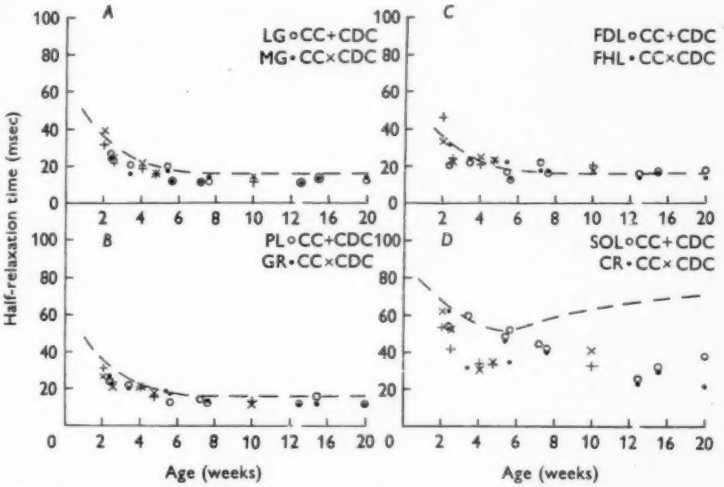

Fig. 10. Half-relaxation times of the muscle twitches; conventions as in Fig. 9. The broken lines show the standard curves as derived in Fig. 4.

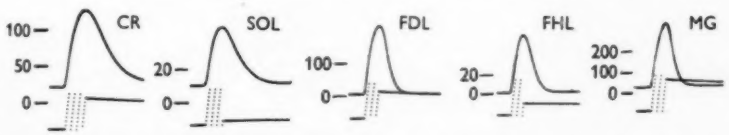

Fig. 11. Twitches for the various muscles in a 33-day-old kitten in which the spinal cord was subjected to an 'isolation' operation, as described in the text, when 4 days old; note the very fast twitches given by crureus and soleus muscles. Weight of kitten, 0·51 kg; tension scales in grams.

plotted in Fig. 10*C*, the half-relaxation times for the FDL and FHL muscles were unaffected by the spinal transection, with mean values of about 17 msec over the same range of age. The early step on the gracilis twitch appears when there is a slack component (R. M. Eccles & Iggo; unpublished).

The stimulus intervals for half tetanic summation of fast muscles also were not significantly affected by the spinal transection.

Severance of all inflow of impulses into lower lumbar spinal cord

A more complete isolation of the lumbo-sacral cord was accomplished in five kittens by an initial operative procedure of eliminating all afferent input into the spinal cord by severing the dorsal roots extradurally on both sides of the stump of cord below the transection in the upper lumbar region, the ventral roots being left intact. This operation has previously been employed in order to effect a complete suppression of the discharge from all motoneurones, so producing disuse atrophy of the muscles (Tower, 1937*a*, *b*, 1939; Eccles, 1941, 1944). Kittens less than 1 week old adapted to this severe operation much better than older kittens. These completely inactivated muscles showed a remarkable gain in weight and power, so that over the first 5 weeks they did not fall below two-thirds of normal in these respects.

The final experimental tests (cf. Fig. 11) revealed that the differentiation of fast muscles proceeded along virtually the normal time course (crosses in Figs. 9*A–C*, 10*A–C*), though, just as with the transected spinal cord, there was possibly a little deviation towards shortening at the longest post-operative intervals. However, with soleus and crureus the contraction times, half-relaxation times and the stimulus intervals for half tetanic summation showed an even more rapid shortening over the first few weeks (cf. Fig. 11 at 33 days old) than was observed after the spinal transection (cf. crosses with circles in Figs. 9*D*, 10*D*). As a consequence, the time courses of differentiation of these muscles (Figs. 9*D*, 10*D*) differed very little from those of fast muscles (Figs. 9*A*, *B*, *C*; 10*A*, *B*, *C*).

Cutting of dorsal roots

Since complete isolation of the spinal cord from incoming impulses produced a larger change in the slow-muscle differentiation than mere section of the spinal cord, it was of interest to see if muscle de-afferentation *per se* would cause any change in muscle differentiation. The dorsal roots of the lumbosacral spinal cord were transected extradurally from L4 to S2 on one side, the other side being left as a control.

Two kittens were tested at 12 weeks after such an operation had been performed 2–3 days after birth. The contraction times were found to be virtually identical on the two sides for the fast muscles, as also were the half-relaxation times and the stimulus intervals for half tetanic tension. In contrast, for the de-afferented slow muscles, soleus and crureus, the mean values for all these three times were 10 to 15% lower than the control values. More investigation is desirable, but it seems likely that de-

afferentation of slow muscles causes a small effect of the same kind as that observed with cord section or isolation of the spinal cord.

DISCUSSION

In kittens a few days old the operative isolation of the lumbosacral spinal cord from all incoming impulses has resulted in completely flaccid hind-limbs. As with adult animals (Tower, 1937*a*, *b*, 1939; Eccles, 1941), there has apparently been an absence of all discharge of impulses from the motoneurones. Under such circumstances it was remarkable that the muscles developed so well in size and in power of contraction. Furthermore, the development of speed of contraction of fast muscles followed a normal time course over the first few weeks (crosses in Figs. 9*A*, *B*, *C*; 10*A*, *B*, *C*); thereafter the contractions were rather faster than normal, particularly during the relaxation phase (Figs. 10*A*, *B*). It can be concluded that the normal process of differentiation of fast muscles occurs independently of their activity. As would be expected, the differentiation of fast muscles was also unaffected by partial isolation of the lumbosacral spinal cord from incoming impulses, either by transection of the spinal cord (circles in Figs. 9*A*, *B*, *C*; 10*A*, *B*, *C*), or by de-afferenting it on the side of the muscles being investigated.

It is of great interest that the slow muscles, soleus and crureus, exhibited such a different behaviour. There was a complete failure of the late phase of slowing of contraction and relaxation (Figs. 9*D*, 10*D*), which normally resulted in the full differentiation of the slow muscles during the age range of 5–16 weeks (Figs. 3*D*, 4*D*). Nevertheless, soleus and crureus remained throughout a little slower than the fast muscles, particularly in their relaxation phase. It seems likely, therefore, that they were subjected to a small differentiating influence even after the operation of complete isolation of the spinal cord. Over the age range of 3–8 weeks the contraction times and half-relaxation times for soleus and crureus tended to be briefer after the operation of complete isolation of the spinal cord than when there was merely a cord transection (compare crosses with circles in Figs. 9*D*, 10*D*). Possibly the spinal transection did not accomplish a full removal of all of the differentiating influence that is normally exerted on soleus and crureus. It seems that de-afferentation is still less effective in removing this differentiating influence, but further investigation is desirable.

The operative transection or isolation of the spinal cord in addition depressed the summation of contractions both of fast and slow muscles. This effect has been observed to develop within 3 weeks of the isolated cord operation in adult cats, the tetanus:twitch ratio being usually reduced to about half the normal value (Eccles, 1941). A similar reduction of the tetanus:twitch ratio was observed when the spinal cord isolation or

transection was performed on 5-week-old kittens (Buller *et al.* 1960). In the adult cat daily stimulations of the muscle caused little or no improvement in the tetanus:twitch ratio, in contrast to the considerable improvement in disuse atrophy that was produced, particularly in flexor muscles (Eccles, 1941). It does not, therefore, seem justifiable to attribute the low tetanus:twitch ratios simply to disuse of the muscles. Yet it is to be distinguished from the effects of the spinal cord operations on the speeds of muscles, for it occurs with fast as well as with slow muscles.

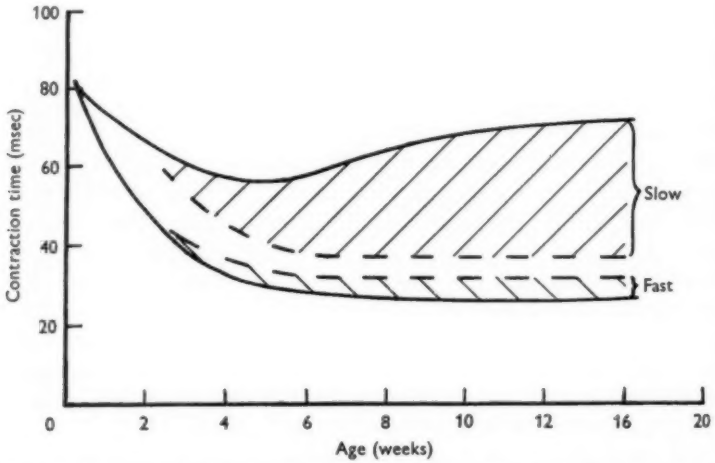

Fig. 12. Diagram showing the standard time courses of the contraction-time–age curves (Fig. 3) for slow and fast muscles (continuous lines), and the postulated curves for these muscles in the absence of neural influences (broken lines). The hatched areas thus give the effects of the respective neural influences.

Under all experimental conditions the contractions of all muscles except gracilis have indicated that there has been no appreciable admixture of muscle fibres with very different contraction and relaxation times. Evidence of an admixture of fast and slow muscle fibres has been obtained for tibialis anticus (Gordon & Holbourn, 1949; Gordon & Phillips, 1953) and for caput medialis of triceps brachii (Eccles *et al.* 1958). It might appear that with gracilis there is an extremely fast component with a contraction time of about 5 msec (Fig. 8 GR). However, recent investigation (R. M. Eccles & Iggo, unpublished) has shown that this effect arises on account of the relative slackness of a component of gracilis that is inserted into the fascia over the gastrocnemius.

It will be convenient to introduce at this stage a diagram (Fig. 12) illustrating the postulated action of differentiating influences on slow and

fast muscles, though some of the supporting evidence is given in the next paper (Buller *et al.* 1959). The two continuous lines are the standard curves for the differentiation of slow and fast muscle contraction times (Fig. 3). The broken lines give the postulated curves when the slow and fast differentiating influences have been eliminated by the operation of the isolated spinal cord. The broken line for slow muscles is that observed in Fig. 9*D*, and the effect of the differentiating influence is portrayed by the hatched area. With fast muscles the isolated cord operation failed to reveal any comparable influence (Fig. 9*A–C*), but when the isolated cord operation was performed on 5-week-old kittens, the fast muscles had contraction times about 5 msec slower than normal (Buller *et al.* 1960, Fig. 11*A*). Possibly the action of disuse during the first few weeks of differentiation had prevented the appearance of these slightly slowed contraction times in Fig. 9. Certainly the small hatched area representing a fast differentiating influence is based upon much less secure evidence than is the slow differentiating influence. Figure 12 also depicts, by the difference between the two broken lines, the small factor of slowness or fastness that remains to characterize the slow and fast muscles after isolation or transection of the spinal cord (Figs. 9, 10); and which is also evidenced by the cross-union experiments of the next paper (Buller *et al.* 1960).

In one respect Fig. 12 may be related to earlier experimental observations. The suggested absence of a differentiating influence from the spinal cord with kittens under 2 weeks old conforms with the general finding of experimental embryology that nerves do not exert a formative influence until late in embryonic life (Hamburger & Levi-Montalcini, 1950; Singer, 1952). The same independence of peripheral tissues has been demonstrated by the good development of muscles, both in weight and contraction tension, that have been completely inactivated from a few days after birth.

The experimental investigations of Gasser & Hill (1924) and of Hill (1949, 1953) on the frog sartorius have indicated that the 'active state' of the muscle contractile process is no greater for a fused tetanus than for a twitch. The recorded 'isometric' contractile tension of the twitch is so much lower because the series-elastic components of the muscle allow shortening even under very rigid extrinsic conditions of isometricity (Hill, 1949; Jewell & Wilkie, 1958); and, as a consequence of the shortening and the muscle viscosity, the rising phase of the twitch contraction is delayed and reaches its maximum only when the 'active state' has subsided to a fraction of its maximum (Hill, 1949) that depends on the particular muscle being studied. On the other hand, during a tetanus at a sufficiently high frequency, the 'active state' is maintained at a steady level, which

gives the tetanic tension during a maintained plateau. No attempt has yet been made to determine the time courses of the 'active states' during twitches of mammalian fast and slow muscles. However, since these muscles exhibit similar differences in time course, not only in contraction time (Figs. 3, 9), but also in half-relaxation time (Figs. 4, 10) and in the stimulus intervals for a corresponding summation of tetanic responses (Fig. 6), it may be assumed that the time courses of their 'active states' are involved in the differences in their speeds of contraction. From the difference in the rate of rise of tension in a tetanus, it appears that the force–velocity relation may also be implicated. Thus the normal differentiation into slow and fast muscles and the changes produced in this by the operative procedures on the spinal cord arise on account of changes in the durations of the 'active states' and in the contractile process of the muscles. The techniques developed by Macpherson (1953), by Macpherson & Wilkie (1954), and by Ritchie (1954) should be employed in order to analyse these differences for the fast and slow muscles under the various conditions of investigation.

SUMMARY

1. Isometric twitch and tetanic responses of hind-limb muscles of cats have been recorded over a wide range of ages, from within one day of birth to the adult. Precautions have been taken to ensure that the responses have been compared under standard conditions of temperature (37–38° C) and initial tension.

2. Earlier observations have been confirmed in that all muscles are equally slow in the new-born animal, and the fast muscles attain their adult speed in about 6 weeks. The slow muscles, soleus and crureus, also quicken, but to a lesser extent, over the first 5 weeks. Thereafter there is a progressive slowing to approach the adult condition in 16–20 weeks.

3. Virtually the same time course for this differentiation of speed is given by the three measurements that have been employed: time to summit of twitch; time from summit to half-relaxation of twitch; interval between stimuli at the tetanic frequency building up to half the maximum tetanic contraction.

4. The differentiation of fast muscles is virtually unaffected by spinal cord transection or by operative isolation of the lumbosacral spinal cord from all incoming impulses. However, the differentiation of slow muscles is greatly depressed, there being a complete failure of the late phase of slowing, so that in a few weeks soleus and crureus become nearly as fast in every respect as normal fast muscles.

5. These results indicate that the differentiation of slow muscles is largely effected by neural influences exerted from the spinal cord.

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INTERACTIONS BETWEEN MOTONEURONES AND MUSCLES IN RESPECT OF THE CHARACTERISTIC SPEEDS OF THEIR RESPONSES

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In the preceding paper (Buller, Eccles & Eccles, 1960) evidence was presented which suggested that the differentiation of slow muscles of the cat hind limb to a large extent failed to occur after certain operative procedures on the spinal cord. This finding indicates that in some way the central nervous system controls muscle differentiation. A more analytic investigation into this postulated influence of nerve on muscle has been accomplished by dividing and cross-uniting nerves to fast and slow muscles, so that motoneurones formerly innervating the fast muscle come to innervate the slow muscle by virtue of the regenerative outgrowth of their fibres, and vice versa for the motoneurones of the slow muscle. The effect of this crossed innervation on the speed of muscle contraction has been tested at varying times after the cross-union. These investigations have also been carried out on animals subjected to the operative procedures on the spinal cord, as previously described (Buller *et al.* 1960). Cross-union experiments allow in addition an investigation into the possible effects in the reverse direction, i.e. of speed of muscle contraction on the conduction velocity of the axons and the after-hyperpolarization of the motoneurones which innervate it. A preliminary account of part of this investigation has already been published (Buller, Eccles & Eccles, 1958).

METHODS

Most of the cross-union operations have been performed on kittens 2–3 weeks old, but some operations had a wide age dispersion: on kittens as young as 6 days; on kittens from 3 weeks to 4 months; and on full-grown cats at least 6 months old. The operations were performed on anaesthetized animals with full aseptic precautions. Usually the cross-union has been effected between the nerves to soleus and flexor digitorum longus muscles (FDL), but there have also been cross-union experiments between the nerves to peroneal muscles and soleus and between the nerves to gracilis and crureus. The position of the nerve suture has been as far distal as possible from the locus of the nerve cross and as far as possible

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from the muscle originally innervated by the nerve. This precaution was important in order to ensure that there was virtually full cross-regeneration uncontaminated by self-regeneration. However, in several of the early experiments a soleus nerve united to the peripheral stump of flexor digitorum longus nerve also achieved a small reinnervation of the medial side of soleus muscle. With the nerve pairs used it was possible to approximate the cut ends without appreciable traction. The respective proximal and distal stumps were then tied together by a very fine strand of nylon thread and the nerve sheaths were opened so that they would not impede the outgrowth of nerve fibres from the proximal stump into the distal stump. In about half the experiments the nerves were similarly divided on the control side, but each proximal stump was reunited to its own distal stump. The operations are illustrated diagrammatically in Fig. 1.

The investigations of muscle contractions were identical with those described in the previous paper (Buller *et al.* 1960). The nerves to the muscles were dissected out as far proximally as possible and cut centrally. It was particularly important to stimulate the cross-sutured nerves well proximal to the site of suture so that there could be no doubt that the muscle contraction was evoked by the motor fibres that originally supplied a particular muscle, e.g. that FDL motor nerve fibres were evoking a contraction in soleus muscle. No complications arise on account of regenerating afferent nerve fibres because they do not make effective motor connexions with muscle fibres (Gutmann, 1945, 1958; Weiss & Edds, 1945; Guth, 1956). In several experiments the conduction velocities of the various motor nerve fibres were measured by stimulating the muscle nerves and recording from the appropriate ventral roots, as described by Eccles, Eccles & Lundberg (1958). Finally, the time courses of after-hyperpolarizations of motoneurones were measured by the standard technique of intracellular recording (Eccles *et al.* 1958).

RESULTS

Nerve cross-union and muscle speeds

Comparison of Fig. 1*B* with *A* shows the invariable result of the cross-union operation: a considerable acceleration of the contraction of soleus muscle reinnervated by FDL nerve fibres, and, complementarily, a considerable slowing of the contraction of the FDL muscle reinnervated by soleus nerve fibres. The soleus and FDL in Fig. 1*A* and *B* had had an equivalent history of nerve degeneration and regeneration. The relative changes in contraction times must therefore arise on account of the nerve cross-union. There were comparable contraction times on the two sides of all the muscles with undisturbed innervation, only two being illustrated in Fig. 1. Not only was the contraction time of soleus shortened to 42 msec in Fig. 1*A*, as compared with the 60 msec for soleus after self-regeneration in Fig. 1*B*, but the time from summit to half-relaxation was correspondingly shortened to 45 from 62 msec. Complementarily, the FDL contraction times and half-relaxation times were lengthened from 33 to 59 msec and from 32 to 67 msec respectively. It will be seen that the two control muscles had twitch tensions within a few per cent of each other on the two sides. In contrast, the cross-innervated muscles developed only about half the twitch tension given by the corresponding self-reinnervated muscles on the control side.

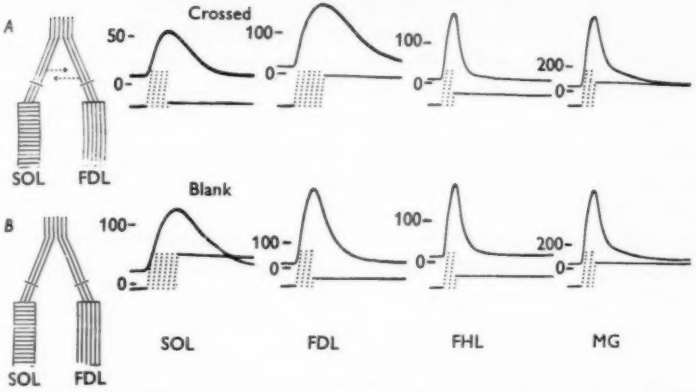

Fig. 1. Series of twitches for the muscles indicated by the symbols to show effect of nerve cross-union: SOL = soleus; FDL = flexor digitorum longus; FHL = flexor hallucis longus; MG = medial gastrocnemius. *A* shows the contractions for the left side in which the soleus and FDL nerves had been cross-sutured (see diagram to left) at the age of 21 days, the experimental investigation being 30 days later. In the control side, *B*, the soleus and FDL had been severed and rejoined at the original operation, as in diagram to left. The decade-counter device (lower beam) gives the contraction times in milliseconds. Weight of kitten = 0·85 kg. Tension scales in grams indicate zero tension.

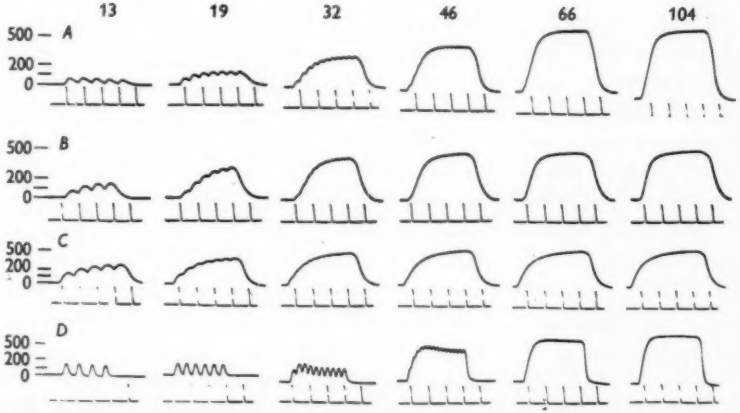

Fig. 2. Repetitive muscle responses to show effect of nerve cross-union. The original nerve operations were performed, as in the diagrams of Fig. 1, on the 16-day-old kitten, and 60 days later the repetitive muscle responses at the indicated frequencies were recorded for the soleus and FDL muscles on the two sides. In the *A* series the soleus muscle was activated by the FDL nerve, and in the *B* series by the soleus nerve, as for soleus in Fig. 1 *A* and *B* respectively. Likewise in the *C* series FDL muscle was activated by the soleus nerve, and in the *D* series by the FDL nerve, as for FDL muscle in Fig. 1 *A* and *B* respectively. Weight of kitten = 1·4 kg. Note tension scales in grams and time scales in 100 msec.

As would be expected, there was an equivalent change in the summation of contractions produced by repetitive stimulation (Fig. 2). The soleus innervated by FDL motoneurones (*A*) required a considerably higher frequency of stimulation to give an equivalent fusion of contractions than in *B*, and conversely with the repetitive responses (*C*) of the FDL muscle innervated by soleus motoneurones. Plotting of the frequency–response curves (cf. Buller *et al.* 1960, Fig. 5*F–J*) shows that in Fig. 2 the stimulus interval for a contraction tension midway between the twitch and the maximum tetanus was shortened from 57 to 28 msec for the FDL-innervated soleus muscle and lengthened from 25 to 65 msec for the soleus-innervated FDL.

It has been regularly observed (cf. Fig. 2) that the ratio of maximum tetanic tension to twitch tension was abnormally high for soleus muscles reinnervated by FDL nerves. The values ranged from 7 to 16, as against normal values of 3 to 8 for self-reinnervated soleus muscles. In contrast, the ratios were in the range 2·5–4·5 for FDL muscles with either self- or cross-reinnervation, which is the range normally observed for both fast and slow muscles of the hind limb (cf. Cooper & Eccles, 1930). However, gracilis always gave exceptionally high ratios, usually 5–7, presumably on account of the summation of the extremely fast component (cf. Buller *et al.* 1960).

The plotted points (open circles) in Fig. 3*A*, *B* give the contraction times of FDL and soleus muscles for the whole series of experiments in which the nerve cross-union was performed in kittens 16–22 days old, with control self-reinnervation on the other side (filled circles). Thus in each case the arrow gives the change in contraction time attributable to the alien innervation. Invariably, soleus reinnervation substantially slowed the FDL muscle, whereas FDL reinnervation quickened soleus muscle. In Fig. 3*A*, *B* there is no indication of a trend in the magnitude of the change with increasing duration of the alien innervation, there being as large a change at 30 days post-operatively as at 90–100 days. However, the series is distorted by the values at 10–16 weeks from three kittens of the same litter that had abnormally fast soleus muscles. Moreover, further investigation is required at shorter intervals post-operatively. So far 23 days has been the briefest post-operative test period (crosses through circles in Fig. 3*A*, *B*), and even then the changes appeared to be as fully developed as with longer test periods.

In general, the plotted points in Fig. 3*A*, *B* show that the effect of the alien innervation falls short of a complete transformation of the muscle, the transformed FDL being faster than soleus muscle at an equivalent age, as indicated by the broken line in *A*; while the transformed soleus likewise remains a little slower than a fast muscle, as indicated by the broken line in *B*. One possible explanation of this incomplete transformation would be that the operation was performed on kittens 16–22 days old when there had already been a considerable differentiation into the fast and slow types

(cf. Buller *et al.* 1960). However, two further investigations eliminated this explanation: the transformation was similarly incomplete when cross-union was performed on 6-day-old kittens (Fig. 4); and there was just as effective a transformation when the cross-union was performed on full-grown cats (Fig. 5). The plotted points to the extreme right of Fig. 3*C*, *D* are the means for the three experiments in which the cross-unions were performed on full-grown cats (over 6 months). With all the experiments plotted in Fig. 3*C*, *D* there was no control operation on the other side. With all the points for ages in excess of 16 weeks the post-operative test periods were very prolonged (100–200 days), but even then the transformations were in general incomplete.

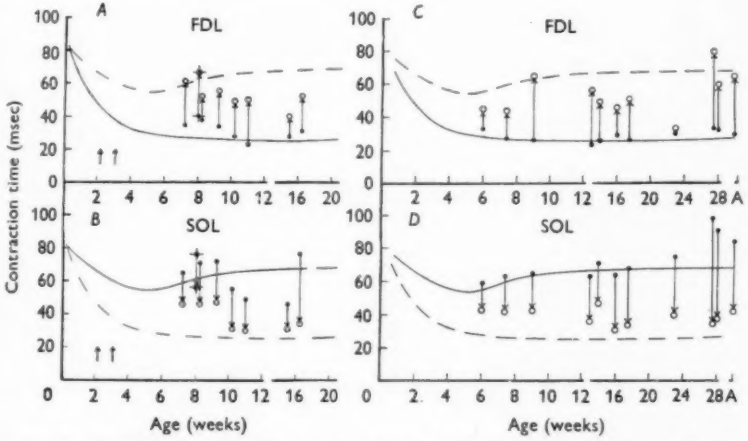

Fig. 3. Plotting of muscle contraction times to show changes produced in all experiments on cross-union between soleus and FDL nerves. Ordinate, muscle contraction times from records such as those of Fig. 1 plotted against abscissa, age in weeks. *A* and *B* give contraction times for FDL and soleus muscles for kittens that were operated at 16–22 days old (age range indicated by arrows), the open circles and filled circles giving respectively the contraction times for the alien-innervated and control muscles as in the diagrams to the left of Fig. 1. The arrows give the direction and magnitude of the change produced by the cross-union. The standard curves for contraction time–age (Buller *et al.* 1960, Fig. 3) are shown as a continuous line for the muscle itself and a broken line for the muscle belonging to the nerve that now provides its innervation; note that the direction of the change is always towards the curve characteristic of the new innervation. The crosses distinguish the points of the 8-week old animal, because that cross-union operation was performed on an older animal (4½ weeks) than the others.

In *C* and *D* the open circles have a similar significance as in *A* and *B*, but the filled circles give contraction times for muscles on which no control operation had been performed, i.e. on muscles that had retained their normal innervation throughout. The points plotted for the adult (marked A on abscissae) are the means of three experiments, one of which is illustrated in Fig. 5.

It is evident enough from the muscle twitches illustrated in Figs. 1, 4, 5 that the cross-union brings about a change in the relaxation time as well as in the contraction time. This is illustrated in Fig. 6*A*, *B*, where times for half-relaxation have been plotted with the same conventions as for the contraction times in Fig. 3. There has been a pooling in Fig. 6*A*, *B* of the measurements for all experiments plotted in Fig. 3*A–D*, and the standard

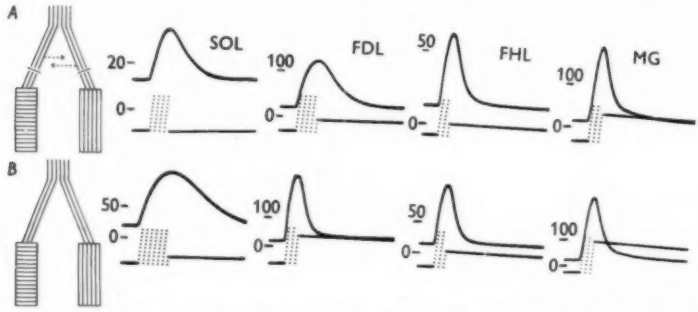

Fig. 4. *A*. Series of muscle twitches as in Fig. 1*A*, but the operation was performed on a 6-day-old kitten and the experimental observation was made 45 days later. As shown in diagram to the left, there was no control operation on the other side, i.e. the *B* series of records are for muscles with undisturbed innervation. Weight of kitten = 0·57 kg. Tension scale in grams.

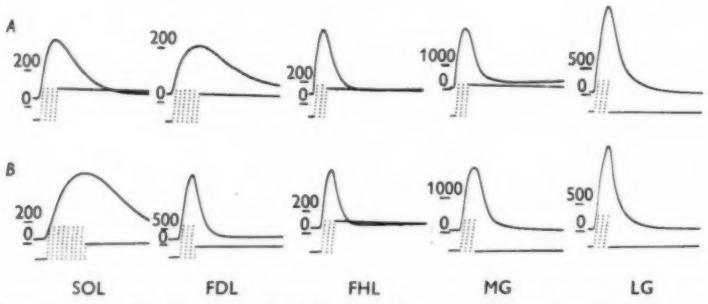

Fig. 5. As in Fig. 1, but the initial cross-union operation was performed on an adult cat; and as in Fig. 4 there was no control operation on the other side. The final experimental test was made 201 days after the operation. Weight of cat = 2·8 kg. Tension scale in grams.

curves for normal muscles (cf. Buller *et al.* 1960, Fig. 4) have been drawn as in Fig. 3. As indicated by the arrows, there has been with only one exception a considerable transformation of all muscles with alien nerve supply, but again the transformation has been incomplete. The very small lengthening observed for FDL muscle at the 23-week experiment (cf. Fig. 3*C*) remains unexplained.

The repetitive responses illustrated in Fig. 2 reveal that the transformation also obtains for the tetanic responses of muscle. This is illustrated in Fig. 6*C*, *D* for all the cross-innervated muscles that were tetanically stimulated. As has been described previously (Buller *et al.* 1960), the time factor for tetanic summation has been measured as the stimulus interval at which the tetanic tension lies midway between the twitch and maximum tetanic tensions. By this criterion, there has been a considerable transformation of all the cross-innervated muscles, but again it tended to be incomplete.

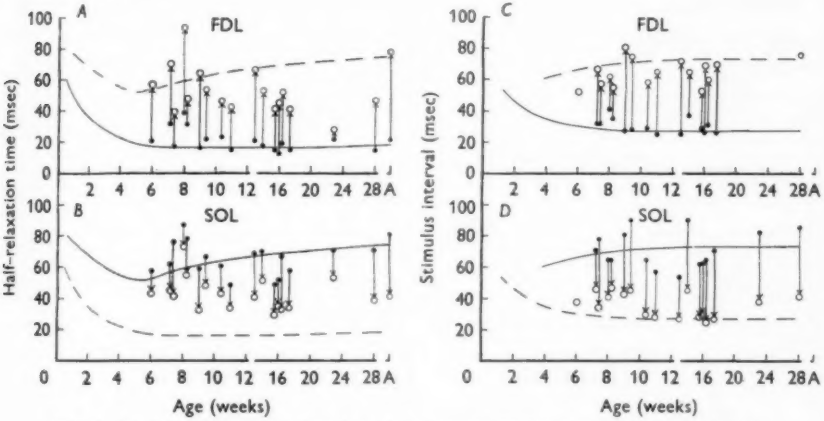

Fig. 6. Plotting as in Fig. 3, but for other measurements on muscle contraction to show effects of nerve cross-union. Half-relaxation times of the muscle twitches corresponding to the two series of Figs. 3*A*, *B* and *C*, *D* are pooled in Fig. 6*A*, *B*. The standard curves, plotted as continuous and broken lines as in Fig. 3, are those determined for half-relaxation times of normal muscles (Buller *et al.* 1960, fig. 4). In *C* and *D* there are plotted as ordinates the stimulus intervals at which repetitive stimulation gave half of the full tetanic summation. Again the standard curves are those determined for the normal muscles (Buller *et al.* 1959, fig. 6).

A possible explanation of the slowing of the FDL contraction when reinnervated by soleus nerve fibres (Figs. 1, 4, 5) would be that impulses in the regenerated soleus nerve fibres were evoking repetitive discharges from the FDL motor end-plates. Single nerve volleys evoke repetitive discharges from motor end-plates in which the cholinesterase has been inactivated (Brown, Dale & Feldberg, 1936; Brown, 1937), and even from the normal motor end-plates in the unanaesthetized preparation (Eccles & O'Connor, 1938; Feng, Li & Ting, 1939), but such repetitive discharges have not been observed in preparations anaesthetized as in the present investigations (Eccles & O'Connor, 1939). In addition, there are two reasons why repetitive discharges cannot account for the lengthening of the FDL contractions

in Fig. 1*A*: the muscle action potential showed no trace of repetitive discharge (Fig. 7*B*); when neuromuscular transmission was blocked by curare, the muscle twitches evoked by direct stimulation of the muscle fibres showed a similar difference when compared with the twitches of the control muscle. For example, in Fig. 7*G* the FDL twitch was throughout much longer than the control FHL twitch (Fig. 7*F*) of the same curarized preparation, 45–27 msec for the respective contraction times and 49–17 msec for the half-relaxation times, which were virtually the same ratios as before the curarization (Fig. 7*C*, *A*). The responses to repetitive stimulation (Fig. 2*C*) further show that the prolongation of the FDL twitches

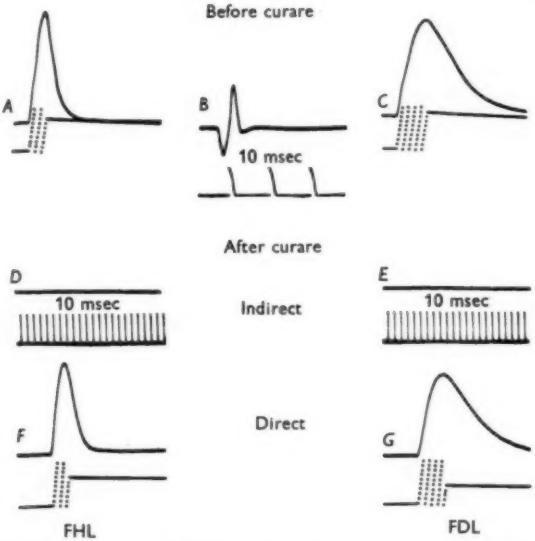

Fig. 7. *A* and *C* are twitches of FHL and FDL muscles with contraction times of 27 and 48 msec respectively, the FDL being slowed by cross-union with soleus nerve. *B* is the action potential led from the surface of FDL muscle. *D*, *E* show that after intravenous administration of curare nerve stimulation failed with both muscles; but direct stimulation was effective in evoking twitches, *F*, *G*, comparable with *A*, *C*. Weight of cat = 2·8 kg.

was not due to repetitive end-plate discharge, for the prolongation was just as prominent with repetitive stimulation, which is contrary to the observations on repetitive discharge from unanaesthetized motor end-plates (Eccles & O'Connor, 1938; Feng *et al.* 1939).

Since the prolonged twitches of FDL muscle fibres innervated by the regenerated soleus nerve were observed even with direct stimulation of the curarized muscle, it must be concluded that the contraction mechanism of

the muscle has been slowed. It must likewise be concluded that the contraction mechanism of the soleus muscle has been quickened on account of its regenerated innervation from FDL motoneurones.

There has been sufficient investigation with other nerve-crossing experiments to allow generalization of the observations with soleus-FDL crossing to the crossing of any combination of nerves to fast and slow muscles. For example, Fig. 8*B* shows the slowing of twitches of gracilis muscle (contraction times lengthened from 26 to 56 msec) when reinnervated by the slow crureus motoneurones, while in Fig. 8*A* the contraction time of the crureus muscle was shortened from 78 to 37 msec when it was reinnervated by the fast gracilis motoneurones. Similarly the twitch of soleus muscle was shortened from 94 to 36 msec when it was reinnervated by motoneurones belonging to the fast peronei muscles (Fig. 8*C*). Thus in general it

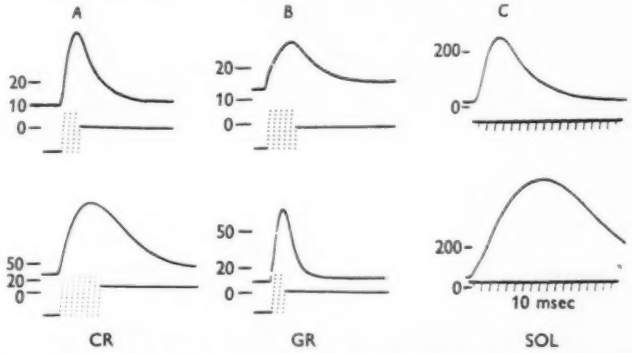

Fig. 8. *A*, *B*, twitch contractions of gracilis and crureus muscles cross-innervated from crureus and gracilis nerves respectively in the upper record; below, the normally innervated control muscles on the other side. The operation was performed on the 22-day-old kitten, and the subsequent experimental test 42 days later; weight of kitten = 0·63 kg. *C*, twitch contractions of soleus muscle, the upper record 180 days after cross-union with the nerve to the peronei muscles (operation on 19-day-old kitten), the lower record being the control from the other side with unmolested innervation. Weight of cat = 2·4 kg.

can be concluded that the regenerated nerve fibres change the speed of the muscle contraction so as to bring it into an appropriate relationship with the frequency characteristics of the innervating motoneurones (cf. Eccles *et al.* 1958). The cross-union experiments further establish that this neural influence is continuously exerted even in the adult. For example, experiments such as that illustrated in Fig. 5 show that the adult soleus motoneurones are very effective in slowing the speed of a fast muscle which they reinnervate, and at the same time the soleus muscle deprived of their influence becomes a fast muscle.

The neural influence is sharply restricted to the muscle fibres that receive the alien reinnervation. For example, in Fig. 9*A* the twitch contraction time of that part of the crureus muscle supplied by a branch from the vastus medialis nerve has been shortened from 77 to 30 msec by reinnervation from the fast gracilis motoneurones. In contrast there was no significant difference for that part of the same crureus muscle with unchanged innervation by the branch from vastus lateralis nerve (Fig. 9*B*). A similar experiment is illustrated in Fig. 9*C–E*, where in *C* the FDL muscle with alien innervation from soleus nerve had a twitch contraction time of 72 msec in contrast to the value of 34 msec for that part with its normal innervation. There was a simple arithmetical summation of the two contractions when evoked in sufficiently close temporal sequence (Fig. 9*D*), as may be seen by comparison with Fig. 9*E*, where comparable wave forms were produced by an electrical model that was designed for arithmetical summation.

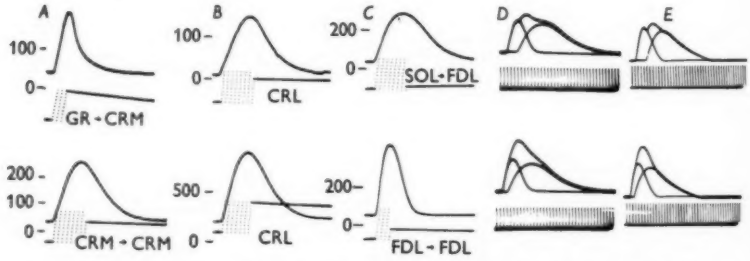

Fig. 9. *A*, *B*, twitch contractions of crureus muscle evoked by the branches from the nerves to vastus medialis (CRM) and lateralis (CRL) respectively, the upper records being from the left limb and the lower from the right. The left gracilis nerve had been cross-sutured to the crureus branch from vastus medialis nerve 150 days previously and consequently there is a fast twitch in the upper record of *A*. The other three contractions were given by the crureus muscle with unmolested innervation. Weight of cat = 3·5 kg. *C*, *D*, contractions of left FDL muscle in another animal. One branch only of FDL had been cross-sutured to soleus nerve at operation on the 6-week-old kitten 21 weeks previously. Upper and lower records of *C* are twitches evoked in the FDL muscle from the soleus and FDL nerves respectively. *D* shows summation of the slow and fast twitches when partly superimposed. *E* shows similar summation of wave forms generated electronically. Weight of cat for *C*, *D* = 3·4 kg. Note tension scales in grams, giving zero tension. Time scales in 10 msec for *D* and *E*.

Investigation of factors modifying the neural influence on muscle

In the preceding paper (Buller *et al.* 1960) operative isolation of the spinal cord from the remainder of the central nervous system, or, more radically, from the peripheral afferent nervous system as well, was found to result in a failure of the late phase of differentiation of slow muscle.

Apparently under such circumstances there was a serious failure of some neural differentiating influence that was concerned in the slowing of the speed of soleus and crureus contractions. It seems likely that the cross-union experiments give evidence of the operation of the same neural influence. It was therefore of interest to perform the cross-union experiments, when in addition the spinal cord had been operatively isolated.

In four experiments on 4-week-old kittens the standard cross-union was effected between FDL and soleus, with the control operation giving self-regeneration on the other side. One week later the spinal cord from L 2 to S 2 segmental levels was isolated by transection and all dorsal roots of this stump of cord were transected extradurally, care being taken to preserve the ventral roots uninjured. Thereafter the animals required careful

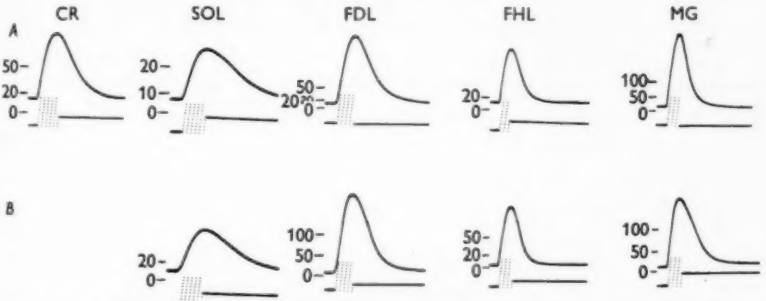

Fig. 10. As in Fig. 1, but with the nerve cross-union and the control operation at age of 28 days. At 35 days the spinal cord was transected above L 2 and below S 2 and all the dorsal roots from L 2 to S 2 were severed extradurally on both sides. Final experimental test when animal was 56 days old. The control crureus twitch was recorded only on one side. Weight of kitten = 0·65 kg. Tension scales in grams giving zero tension.

nursing with bladder and bowel emptying twice daily and skin treatment to minimize the development of bed-sores. All survived in good condition sufficiently long for effective regeneration of the severed nerves and the final experimental investigation of the muscle contractions. As illustrated in Fig. 10, there was under such conditions no appreciable change in the speed of muscle contraction as a consequence of the cross-union that had been effected 31 days earlier. The respective contraction times were 54 and 55 msec for the crossed and control soleus muscles, the half-relaxation times being 81 and 82 msec. Similarly the FDL was virtually the same on the two sides, with contraction times of 41 and 43 msec and half-relaxation times of 44 and 40 msec for the crossed and control muscles respectively. There was also no appreciable difference between the repetitive responses on the two sides.

Comparable results were given in the other three experiments, as illustrated by the plotted points of Fig. 11*A*, *B*. When compared with the cross-union experiments uncomplicated by the 'isolation' operation (Fig. 3*A*, *B*), the FDL contractions on the control side (filled circles in *A*) were slower, while the control soleus contractions (filled circles in *B*) at the two longest test intervals were faster. In part the slowing of the FDL contractions relative to the standard age–contraction-time curve (continuous line in Fig. 11*A*) is attributable to the aftermath of nerve degeneration and regeneration, as is seen in the three control values (filled circles) at 4–6 weeks post-operatively in Fig. 11*A*. However, there was in

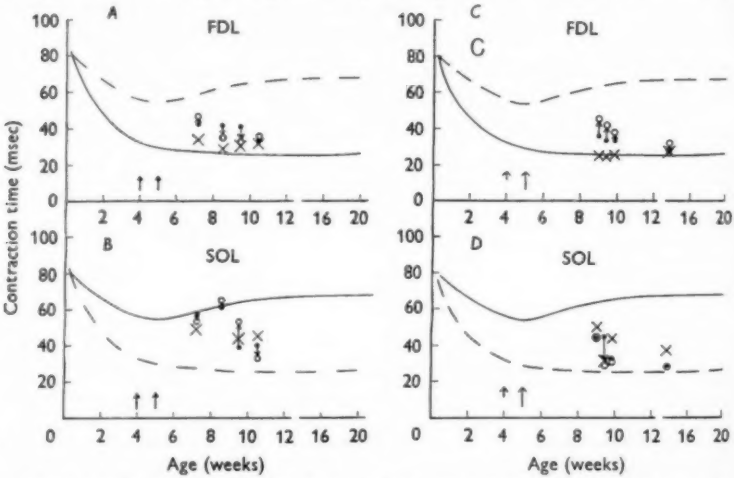

Fig. 11. *A*, *B*, plotting as in Fig. 3 of effect of cross-union on contraction times of FDL and soleus muscles, but with in addition the cord isolating operation as in Fig. 10. The oblique crosses show mean values for the contraction times of those fast and slow muscles, respectively, which remained on both sides with unmolested innervation, LG, MG, PL, FHL in *A* and crureus in *B*. *C*, *D*, as with *A*, *B*, control operation at 28 days, but the subsequent operation at 35 days was limited to transection of the spinal cord at L2 level. Again the oblique crosses give mean contraction times for other muscles. In *A*–*D* the arrows at 4 weeks show time of nerve cross-union operation, and arrows at 5 weeks the time of the later cord operation. The standard curves for the contraction times are drawn as continuous and broken lines with the same conventions as in Fig. 3.

addition a slowing of the fast muscles with unmolested innervation, as is indicated by the oblique crosses lying above the standard curve in Fig. 11*A*, each cross being the average of the contraction times for four fast muscles on each side. Thus it appears that the slowing of the control FDL contraction times in Fig. 11*A* was due to the operation of two factors. Cross-innervation by the soleus nerve fibres gave no additional effect,

there being a slight slowing in two experiments and a slight quickening in the other two.

As would be expected, the soleus observations were complicated by another factor. The control observations on the crureus muscle with unmolested innervation (oblique crosses) reveal the same progressive shortening of contraction time that was observed both with soleus and crureus when the isolated cord operation was performed on younger animals (Buller *et al.* 1960, fig. 9). The contractions of the control soleus muscles with self-regeneration (filled circles) did not differ in any systematic way from these unmolested crureus responses. Thus in Fig. 11*B* the operative isolation of the spinal cord provided the over-riding factor determining the speeds of contraction of the soleus muscles. Cross-reinnervation by the FDL nerve caused no demonstrable additional effect, as is indicated by two experiments giving shortening and two lengthening (note arrows).

A similar absence of transformation by the cross-innervation was also shown by the other indicators of muscle speed, half-relaxation and the stimulus intervals for half-tetanic summation. The values for the individual muscles showed changes comparable with those occurring in the contraction times in Fig. 11*A*, *B*, there being likewise no significant change in the mean values.

When the initial operative lesion was merely transection of the spinal cord in the upper lumbar region, there was evidence of a less complete deprivation of the neural influence responsible for slowing the soleus and crureus contractions (Buller *et al.* 1960, figs. 9. 10). It would, therefore, be expected that after such an operation the cross-union experiment would provide evidence of a neural influence on muscle of the same kind as that normally observed, but smaller in amount. The nerve cross-union operation together with the control operation was performed on kittens 4 weeks old and one week later the spinal cord was transected in the upper lumbar region, i.e. this latter operation was performed well before the reinnervation was established. When the muscle contractions were investigated 4 weeks after the initial operation, it was found that there had been a small transformation of the muscles with alien innervation (Fig. 12). For example the FDL muscle with soleus innervation had lengthened contraction times and half-relaxation times, 36 msec lengthened to 45 msec in both cases, while the soleus muscle with FDL innervation had a faster twitch, the half-relaxation time being shortened from 51 to 35 msec, though the contraction times were both 44·5 msec. The repetitive responses indicated comparable changes, the stimulus interval for half-tetanic summation being lengthened from 33 to 54 msec for FDL and shortened from 62 to 39 msec for soleus. However, as is shown for the plotted points of the

muscle contraction times for the four experiments in Fig. 11*C*, *D*, the muscle transformation was on the average much less than half of that normally observed (cf. Fig. 3*A*, *B*). This relatively small transformation was also shown by the half-relaxation times and stimulus intervals for half-tetanic summation; the respective lengthenings of the mean values were from 32 to 34 msec and from 35 to 41 msec for the cross-innervated FDL muscles; and the respective mean shortenings were from 43 to 28 msec and from 49 to 36 msec for the cross-innervated soleus muscles.

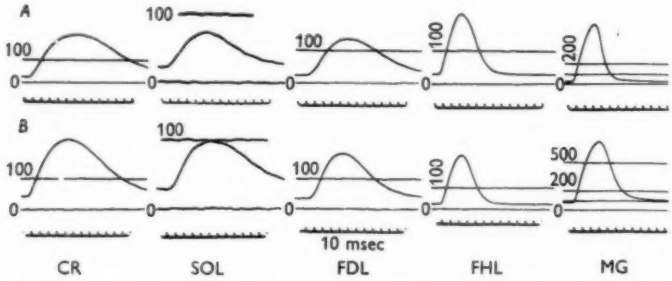

Fig. 12. Series as in Fig. 10, but in which the operation at 35 days was limited to a spinal transection at the L2 level. Measurements from the four experiments of this type are plotted in Fig. 11*C*, *D*. Weight of kitten = 0·85 kg. Note tension scales in grams, giving zero tension.

The muscle influence on nerve

The effectiveness of the neural influence on muscle contraction time has obscured a possible alternative relationship—the influence which muscle may exercise on the time characteristics of the innervating motoneurones. So far no attempt has been made to record intracellularly from motoneurones in young kittens, and so to determine whether there is any process of differentiation of motoneurone speeds to match that observed for muscle (Buller *et al.* 1960); consequently the only experimental evidence relates to motoneurones which on account of nerve cross-union innervate muscles having inappropriate speeds. Since the muscles themselves are so effectively changed by the motoneurones (Figs. 1–6), no large changes could be expected in the reverse direction. Two criteria have been employed in an attempt to detect such a change.

The duration of the after-hyperpolarization following the invasion of a motoneurone by an impulse has uniformly been longer in soleus than gastrocnemius motoneurones (Eccles *et al.* 1958). Five soleus motoneurones have been recorded from intracellularly after they had innervated the fast muscle, FDL, for over 20 weeks. The contraction times of these FDL muscles were of course considerably slowed as a consequence (cf. Figs. 3, 6),

but the after-hyperpolarization showed no complementary quickening, being still uniformly over 150 msec in duration. Small changes would not be detected by this relatively insensitive test. A more sensitive method of detecting small changes is provided by measurements of conduction velocities of motor axons after cross-union experiments, control measurements being provided by the other limb in which the same nerves were severed and then rejoined to their own peripheral stumps. There was, likewise, no appreciable change (less than 3 % in either direction) in conduction velocity as a result of the cross-union, i.e. within the accuracy of measurement soleus nerve fibres had the same conduction velocity on the two sides, as also did FDL nerve fibres. Thus we may conclude that muscle fibres exert no appreciable influence on the time characteristics of motoneurones that innervate them. It is to be noted that this negative result is obtained for the direction of the modulating influence postulated by Wiersma (1931), Weiss (1950) and Sperry (1950, 1951). It can be concluded that the interaction influencing the time characteristics of motoneurones and muscle fibres occurs very largely, if not exclusively, in the reverse direction, from motoneurones to muscle fibres.

DISCUSSION

In the preceding paper (Buller *et al.* 1960) it was shown that the operation of isolation of the spinal cord from all incoming impulses caused a failure of the normal process of differentiation of slow muscles, and a simple transection of the spinal cord was rather less effective in the same way. Exactly parallel results have been obtained when the nerve cross-union operation was employed to test for muscle differentiation, cord isolation resulting in the complete failure of nerve cross-union to change muscle speed (Fig. 11*A*, *B*), while after cord transection it produced only a small change (Fig. 11*C*, *D*). It may be postulated, therefore, that the same neural influences are bringing about the muscle transformation in the normal process of muscle differentiation and in the artificial conditions created by the nerve cross-unions.

The simplest explanation of the influence of motoneurones on muscle speed would be that it is due to the frequency of impulse discharge: the contraction time of a slow muscle is accelerated when subjected to the high-frequency discharges from fast (phasic) motoneurones; while a slowing of contraction time ensues when a fast muscle is subjected to the relatively slow frequencies of discharge from tonic motoneurones. Phasic motoneurones usually discharge at frequencies from 30–60/sec, while with tonic motoneurones the frequency is usually 10–20/sec (Denny-Brown, 1929; Adrian & Umrath, 1929; Granit, Henatsch & Steg, 1956; Granit, Phillips, Skoglund & Steg, 1957).

It would be very difficult technically to arrange artificial stimulation of several weeks duration so that there was a prolonged transposition of these characteristic frequencies of action, independently of nerve cross-union. However, the postulated continuous relationship between frequency of activation and the resultant muscle speed can be tested by eliminating all discharge from motoneurones. This requirement is achieved by the isolated spinal cord operation, as was first demonstrated by Tower (1937*a*, *b*). Under such conditions there is no inflow of impulses into the isolated lumbosacral segments of the spinal cord, and consequently no discharge therefrom, the hind limbs remaining absolutely flaccid. According to the frequency hypothesis it would be predicted that the complete cessation of all discharge would give a more effective slowing of a fast muscle than the low frequency of activation consequent on reinnervation from soleus motoneurones (Fig. 3*A*). But the reverse is observed: the small lengthening of contraction time (about 5 msec above the standard curve) for the oblique crosses in Fig. 11*A* contrasts with the much larger lengthening for the soleus-reinnervated FDL muscle in Fig. 3*A*, *C*, which was about 20 msec longer on the average. Similarly the small lengthenings of the half-relaxation time and of the stimulus interval for half tetanic summation contrast with the much larger lengthenings in Fig. 6*A*, *C*. The frequency hypothesis fails even more significantly in Fig. 11*B* (oblique crosses), where cessation of all motoneuronal discharge to crureus muscle caused a considerable quickening of its contraction (contraction times 10–20 msec shorter than for the standard curve), which is the reverse change from the slowing that would be predicted.

An alternative hypothesis would be that muscle speed was controlled by the aggregate number of impulses activating the muscle fibres in a given time, the higher the aggregate the slower the muscle speed; and that the more continuously active tonic motoneurones would actually be delivering a higher aggregate despite their lower frequency. It certainly would be surprising if the sharp differentiation of muscles into fast and slow types (cf. Buller *et al.* 1960) were effected by such a relatively variable factor as the aggregate number of impulses fired by tonic and phasic motoneurones respectively, particularly when it seems that animals differ so much in their phasic activity. But in addition to this general criticism, the aggregate hypothesis is contra-indicated by the effects of reducing the aggregate to zero. The predicted quickening of slow muscles does indeed then occur (Fig. 11*B*, oblique crosses), but it certainly is no larger than when slow muscles are reinnervated by phasic motoneurones (Fig. 3*B*, *D*). Moreover, the oblique crosses in Fig. 11*A* show that with zero activation fast muscles are slower than normal, whereas the aggregate hypothesis would predict the opposite change.

A third hypothesis has been suggested by Mr A. F. Huxley. A fast muscle would give a grossly oscillatory response at the prevalent frequency of stimulation provided by slow motoneurones (cf. Buller *et al.* 1960, Fig. 5*C–E*, at 13/sec), and conceivably this oscillation could effect the gradual transformation to a slow muscle, so alleviating the stress of the oscillatory contraction. When not acted on by this postulated differentiating influence, i.e. with higher frequencies of stimulation or with zero stimulation, all muscle fibres become fast. Since slow motoneurones vary so much in their frequency of discharge, it seems unlikely that the sharp separation which characterizes fast and slow muscles could be produced by such a frequency-dependent influence. This hypothesis has the virtue that it can be tested experimentally: for example, it would predict that the muscles of a spinal animal would be slowed (in contrast to Fig. 9*D*, Buller *et al.* 1960) if they were artificially stimulated at the postulated frequency of 10–20/sec.

A further possibility is that some specific influence is exerted by the impulses discharged by tonic and phasic motoneurones along their axons. Tonic motoneurones certainly differ in that they have smaller motor axons (Eccles & Sherrington, 1930; Hagbarth & Wohlfart, 1952), which consequently have slower conduction velocities and higher thresholds (Eccles *et al.* 1958) and give smaller spikes when recorded in a nerve trunk (Granit *et al.* 1956, 1957). It is not known if this relative difference between the motor axons in the nerve trunks continues down to the fine terminal branches that make the actual contact with the muscle fibres at the motor end-plates, but in any case it seems inconceivable that it could provide the differentiating factor for muscle speed. Furthermore, it does not seem warranted to postulate some difference in the chemical transmitters liberated at the neuromuscular junctions formed by the tonic and phasic motoneurones respectively. Pharmacological investigations have revealed quantitative differences between the neuromuscular junctions to fast and slow muscles (Paton & Zaimis, 1951; Zaimis, 1954; Jewell & Zaimis, 1954; Bowman & Zaimis, 1958), but these slight differences have not shaken the belief that acetylcholine is the transmitter at both types of junction.

Before attempting to define more precisely the nature of the neural influences responsible for muscle speed, it is expedient to consider the possibilities that the influence is exerted through afferent fibres or sympathetic fibres. The latter possibility can virtually be excluded because the sympathetic innervation of the hind-limb muscles would largely be intact in the experiments illustrated in Figs. 10, 11, where the cord section was at the second or third lumbar segment. However, a more crucial test would be provided by cross-union experiments on animals in which the sympathetic innervation of the hind limb had been eliminated by excision of the sympathetic trunk at the appropriate levels. An influence of afferent fibres on muscle is highly improbable because they do not enter into close relationship with the extrafusal fibres of muscle, which are responsible for all the tension developed in muscle

contraction (Kuffler, Hunt & Quilliam, 1951); and no histological changes occur in muscle fibres when the afferent innervation of muscle is degenerated (Tower, 1931). However, evidence for an influence of the afferent innervation is provided by the finding that after months of de-afferentation the contraction of slow muscles was appreciably faster than on the control side, the fast muscles being unchanged (Buller *et al.* 1960), which is a slight effect of the same kind as with spinal cord transection or isolation. It has been suggested (Buller *et al.* 1960) that de-afferentation produced this effect by diminishing the activity of the tonic motoneurones. A crucial investigation designed to exclude a direct action of afferent fibres on muscle would be to effect cross-unions on the nerves to muscles de-afferented by appropriate ganglionectomies, but this has not yet been done.

When attempting a more critical evaluation of the evidence relating to neural influences and muscle differentiation, it emerges that a neural influence that slows muscle contraction has been indubitably established, whereas the evidence is less decisive for the existence of an opposite neural influence that quickens muscle contraction. For example, after transection or 'isolation' of the spinal cord in the young kitten, the differentiation of fast muscles did not appreciably differ from normal, whereas there was almost complete failure of the differentiation of slow muscles (Buller *et al.* 1960). However, when the isolated spinal cord operation was performed on older kittens (5 weeks), the fast muscles were appreciably slower at 7–10½ weeks of age (Fig. 11*A*, oblique crosses), which suggests that there has been defection of a small quickening influence that is normally exerted by fast motoneurones on muscle. The cross-union experiments probably also provide evidence of a fast neural influence that accelerates the soleus muscle contraction, though conceivably this effect could be entirely due to the deprivation of the slow neural influence. Further investigation is required on the time course of the onset of changes in muscle speeds after nerve cross-union.

The changes that nerve cross-unions effect in muscle contraction times are much larger than can be accounted for by changes in conduction velocity and duration of impulses travelling along the muscle fibres from the motor end-plates. For example, the spike potentials of slow muscles are a little longer than those of fast muscles and their propagation velocity is a little slower; but a transformation of a fast muscle in these respects would account at most for a lengthening of contraction time by 3–4 msec (Eccles & O'Connor, 1939). Thus the muscle transformation must occur not only along the whole length of the muscle fibre, but it must also involve the actual contractile mechanism of each element. As yet there has been no histological investigation of the transformed muscles, but a question of great interest concerns the possibility of a transformation of 'Fibrillinstruktur' to 'Felderstruktur' and vice versa, in accordance with the characteristic fibre patterns described by Krüger (1952) for fast and slow muscle.

The muscle transformations brought about by the nerve cross-unions have been assessed by three different measurements for the speed of muscle contraction, the rising time to the summit of a twitch, the half-relaxation time from that summit, and the stimulus interval for a repetitive stimulation that gives a tetanic contraction half way between the twitch and the maximum tetanic contraction. The similar results given by these three criteria indicate that there has been a fairly uniform transformation of all the fibres of a muscle. Since comparable changes have been observed with these three different measurements both after cross-union (Figs. 3, 6) and also with the normal processes of differentiation of slow and fast muscles (Buller *et al.* 1960, Figs. 3, 4, 6), it can be postulated that the differentiation of slow and fast muscles is brought about by changing the time course of the 'active state' developed in the muscles. Changes in such passive properties as the series-elastic component or in the viscosity of the muscle might cause considerable changes in the time course of a twitch, but not in the summation of tetanic responses at various frequencies. It would be difficult to apply the more precise tests for 'active state' that have been developed by Hill and his collaborators (Hill, 1949, 1953; Jewell & Wilkie, 1958), but investigations of the type described by Macpherson (1953), by Macpherson & Wilkie (1954) and by Ritchie (1954) could be applied in an attempt to provide quantitative measurements of the alterations that nerve cross-unions bring about in the durations of the 'active state'.

It will be convenient now to employ a diagram (Fig. 13) in developing a specific chemical hypothesis of the neural influence exerted by nerve cells on muscle fibres. The postulated neural influence would be exerted by a specific chemical substance that courses along the motor nerve fibres, as shown in Fig. 13, then traverses the neuromuscular junction and finally passes thence along the whole length of the muscle fibres, inducing therein changes that alter the speed of the muscle contraction. There is convincing experimental evidence for a neural influence emanating from tonic motoneurones and slowing muscle contraction. There also may be a different influence emanating from fast motoneurones and quickening muscle contraction, but the evidence for this is much less satisfactory. The hypothesis illustrated in Fig. 13 can account for the absence of neural influence by motoneurones in the 'isolated' and completely quiescent spinal cord (Figs. 10, 11*A*, *B*; cf. also Buller *et al.* 1960) in four different ways: the substance may not be produced in motoneurones or transported thereto in the absence of synaptic bombardment; it may not be able to travel down motor axons in the absence of impulses; it may not be able to cross quiescent neuromuscular junctions; the pumping action of muscle contractions may be required to propel the substance from the motor end-plate region along the whole length of the muscle fibres where it exerts its

transforming action. Experiments are being undertaken to test several of these alternatives. Presumably the mere transection of the spinal cord causes a diminution of the neural influence (Figs. 11 *C*, *D*, 12) by the relative depression of cord activity thereby induced.

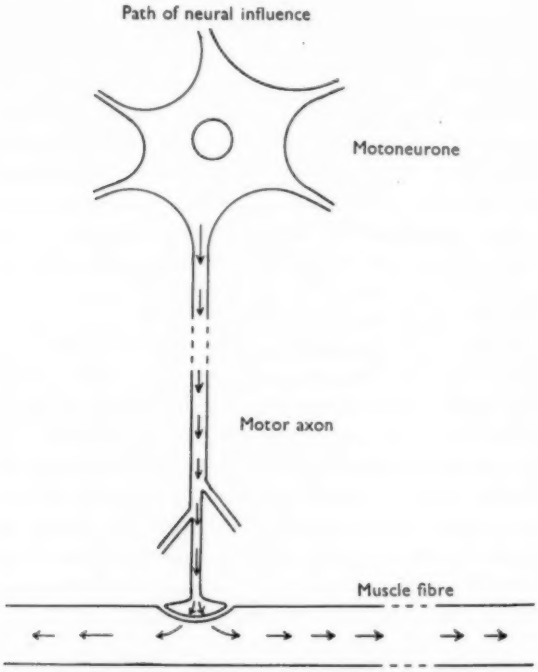

Fig. 13. Diagram to show postulated path for the flow of the slow (and fast) neural influences from the motoneurone down the motor axon and along the muscle fibre.

Since muscles of full-grown cats were transformed just as effectively as those of kittens (Figs. 3, 5, 6), the neural influence must be continuously exerted by nerve cells throughout life. Thus we are introduced to a new concept, that of a continuously operating differentiating principle. Furthermore this principle is exerted by slow motoneurones to slow the contractions of muscles, so appropriately relating their responses to the slow frequencies of discharge of tonic motoneurones. It is therefore of significance in improving the efficiency of operation of tonic motor units. The question thus arises: Are influences of a comparable kind exerted more generally, so enhancing the efficiency of other functional connexions between cells, for example not only from neurone to muscle fibre, but also from neurone to neurone?

Consideration should be given to the possible relationship of these neural influences on muscle contraction time to the 'trophic' influences which have been regarded as a separate phenomenon from the activation of muscle by nerve impulses (Tower, 1937*a*, *b*; 1939; Eccles, 1941*a*, *b*; Luco & Eyzaguirre, 1955). For example the supersensitivity to acetylcholine, the spontaneous fibrillation and certain histological changes occur when the motor nerve is severed, but not when muscle is simply deprived of nerve impulses. It has been found that the first two effects take longer to develop the more central the nerve section (Luco & Eyzaguirre, 1955). Consequently it has been postulated that there is some 'trophic' influence in the motor axon which slowly spreads along it with a speed at most of 2 mm/hr. Axelsson & Thesleff (1959) have shown that after denervation of mammalian muscles sensitization to acetylcholine develops uniformly along the whole length of the muscle fibres, there being no special change at the end-plate region. Apparently this 'trophic' influence of the motor axon operates to restrict the acetylcholine sensitivity to the end-plate region, an effect which is particularly well illustrated by the spreading sensitivity that develops only at the denervated end-plates on muscle fibres with multiple innervation (Miledi, 1959). Moreover, this action is independent of nerve impulses, for the sensitization does not occur in disused muscle. This trophic influence on the surface membrane of the muscle fibre is presumably distinct from the postulated fast and slow neural influences on the contractile mechanism of the muscle fibre.

SUMMARY

1. Nerve cross-union experiments have been employed in the cat hind limb in order to discover if motoneurones determine the speed of muscles, or if the effective influence is in the reverse direction.

2. When a nerve from fast or phasic motoneurones has been made to innervate a slow muscle, the muscle is transformed to a fast muscle, even in the adult; likewise slow or tonic motoneurones convert fast muscles to slow.

3. The transformation of muscle speed is shown not only by the time course of the rising and falling phases of the twitch contraction, but also by the summation of tetanic contractions at various frequencies.

4. The slow or fast muscle with alien innervation has no detectable influence in the reverse direction, i.e. on the motoneurones. The conduction velocity of the motor axons and the time course of motoneural after-hyperpolarization are both unchanged.

5. The muscle transformation falls short of a complete change to the slow or fast type.

6. Isolation of the lumbosacral spinal cord from all incoming impulses, with consequent silence of motoneurones, causes a failure of all transformation by cross-union. Mere transection of the spinal cord greatly reduces the transformation.

7. It is concluded that the neural influence on muscle speed is not exerted by nerve impulses as such. It is postulated that a substance passes down the axons of slow motoneurones, crosses the neuromuscular junctions and traverses the muscle fibres, transforming them into slow contracting units and maintaining them so. Possibly there is also a substance from fast motoneurones that acts via a comparable pathway to accelerate muscle contraction.

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EFFECT OF HYDRAZINE ON THE EXCITABLE MEMBRANE IN SODIUM-FREE MEDIA

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Among the nitrogenous compounds which can replace sodium ions in maintaining electrical excitability, hydrazine seems to be the most favourable (Koketsu, Cerf & Nishi, 1959*b*; Koketsu & Nishi, 1959). Since the action potential of frog spinal ganglion cells could be elicited in sodium-free sucrose solution by injecting hydrazine into the cell-body (Koketsu *et al.* 1959*b*), it would appear that the intracellular hydrazine was responsible for the maintenance of membrane excitability in sodium-free media. Consequently, the possibility that hydrazinium ions may be acting as charge carriers during active membrane depolarization was dismissed (Koketsu *et al.* 1959*b*). The question was then raised as to whether the intracellular hydrazine was also responsible for the generation of spike potentials in the case of frog skeletal muscle fibres.

The present study was aimed primarily at investigating the mechanism of the action potentials of muscle fibres in a sodium-free hydrazine solution. The results indicate that the external rather than the intracellular hydrazine is important for the generation of action potentials of skeletal muscle fibres. The discrepancy between the results obtained with spinal ganglion cells and muscle fibres led the authors to make confirmatory experiments on the effect of the intracellular hydrazine in ganglion cells.

METHODS

Experiments were performed mainly on sartorius muscles and spinal ganglion cells of frogs (*Rana pipiens*) at room temperature (24–26° C). The sodium-free sucrose solution used throughout consisted of (mM) sucrose 224, $KHCO_3$ 2, and $CaCl_2$ 1·8 (pH 6·8–7·0). The sodium-free hydrazine solution was prepared in exactly the same way as has been described in a previous paper (Koketsu *et al.* 1959*b*). Sulphuric acid or nitric acid was sometimes used instead of hydrochloric acid for adjusting the pH of the hydrazine solution. In some experiments the hydrazinium or calcium ions were replaced by equimolar amounts of sucrose. Experimental arrangements for recording intracellular action potentials of muscle fibres and spinal ganglion cells were similar to those described in previous papers (Koketsu, Cerf & Nishi, 1959*a*; Koketsu & Nishi, 1959). The action potential of isolated single muscle fibres was recorded extracellularly by using a pair of large platinum electrodes. The technique of intracellular injection of various nitrogenous compounds into the cell bodies was

similar to that described in previous papers (Koketsu *et al.* 1959*a*, *b*). In the case of muscle fibres, KCl-filled and hydrazine-filled micro-electrodes were used as recording and stimulating electrodes, respectively. After being tested for normal activity in Ringer's solution, the muscles were soaked in sodium-free sucrose solution for 30 min and the spinal ganglia for 60 min in order to eliminate electrical activity, whereupon they were immersed in a sodium-free hydrazine solution.

RESULTS

Isolated single muscle fibre

In this experiment single muscle fibres were isolated from M. iliofibularis. The electrical activity of isolated single muscle fibres was easily eliminated when they were soaked in sucrose solution for 5–10 min. It was promptly restored when the preparations were immersed in 80–112 mM hydrazine solutions (Fig. 1).

Effects of external anions

No appreciable difference in the value of the resting potentials of muscle fibres was observed between the various hydrazine solutions (80–112 mM) containing sulphate, nitrate or chloride ions. The time constant of the resting membrane increased when the chloride ions were replaced by either sulphate or nitrate ions. The electrical activity of muscle fibres was well maintained in hydrazine-nitrate and hydrazine-sulphate solutions, and no appreciable change in the configuration of the spike potentials was observed in these solutions in comparison to that in hydrazine-chloride solution (Fig. 2).

Effects of different concentrations of hydrazinium ions

The resting and action potentials of muscle fibres soaked in 80–90 mM hydrazine solutions were described in a previous paper (Koketsu & Nishi, 1959). In this study the concentration of hydrazine was changed and the relationship between the action potential and the external hydrazine concentration was studied.

The resting potential of muscle fibres in a solution containing 80–90 mM hydrazine was 90 mV after immersion for 10–30 min, and gradually dropped to about 80 mV after 60–120 min (Koketsu & Nishi, 1959). These values were not appreciably altered by changing the concentration of hydrazine from 50 to 220 mM. When the hydrazine concentration was reduced to less than 50 mM the resting potential tended to fall. The value of the resting potential shown in Fig. 3 is an average of twenty fibres, which were first immersed in a 112 mM hydrazine solution for 30 min and then in a solution containing a given amount of hydrazine for another 30–60 min. The values of the electrical constant of the resting membrane in solutions containing 50–220 mM hydrazine did not show appreciable deviations from those reported in a previous paper (Koketsu & Nishi, 1959).

When the muscles were immersed in a 112 mM hydrazine solution the electrical activity was restored within 1 min. If the amount of the intracellular hydrazine which diffused into the muscle plasma were an important factor, dominating the electrical activity, the action potential could be affected by further immersion. No appreciable differences, however, were found by comparing action potentials recorded immediately after, and between 90 and 120 min after, immsersion in a 112 mM hydrazine

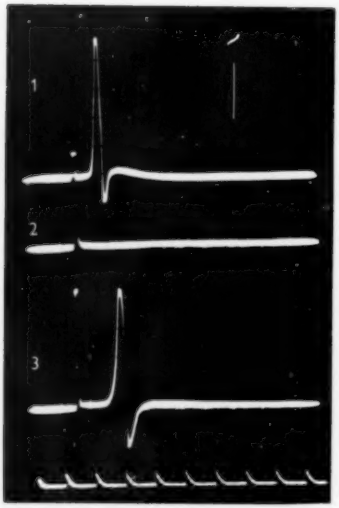

Fig. 1

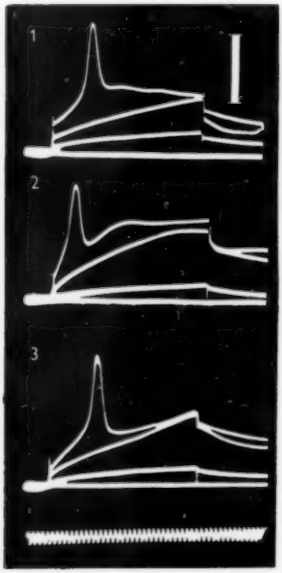

Fig. 2

Fig. 1. Action potentials recorded extracellularly from isolated single muscle fibres in Ringer's solution (1), 10 min after soaking in sucrose solution (2), and (3) 5 min after immersion in 112 mM hydrazine solution. Calibration, 5 mV; time marker, 1 msec.

Fig. 2. Intracellular action potentials of muscle fibres recorded in 112 mM hydrazine solution of which pH was adjusted with HNO_3 (1), H_2SO_4 (2), and HCl (3). Calibration, 40 mV, time marker, 1 msec.

solution. Figure 3 shows the relationship between the peak potential of spikes (active membrane potential) and the external concentration of hydrazine. This result was obtained from a muscle preparation which was first immersed in a 112 mM hydrazine solution for 30 min and then successively immersed in solutions containing 112, 70, 50 and 30 mM hydrazine for 30–40 min, respectively. The active membrane potential decreased according to the decrease of the external hydrazine concentration; the

slope being practically identical with that obtained by the same experimental procedure with Ringer's solution, where the external sodium concentration was altered. In both the sodium and hydrazine solutions the reduction of the concentration to one-tenth resulted in a decrease of the spike height of approximately 58 mV (the value of RT/F at room temperature). A similar relation was observed in muscle preparations which

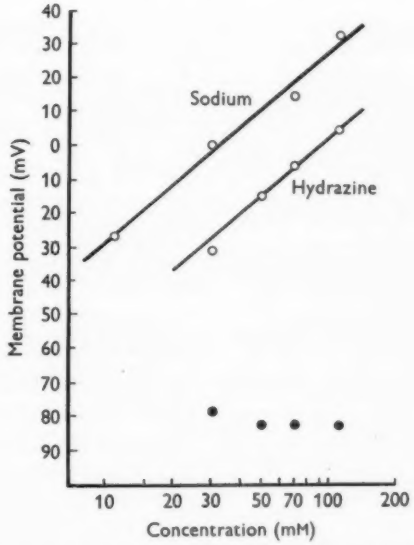

Fig. 3. Relation between active membrane potential and external concentration of sodium and hydrazinium ions obtained from two different preparations according to experimental procedure described in text. ○, average value of active membrane potential obtained from six different muscle fibres of a preparation. ●, mean value of resting potential obtained from six different preparations soaked in hydrazine solutions (for details see text). Semi-log. scale.

were directly immersed in various concentrations of hydrazine for 60–90 min without previous soaking in 112 mM hydrazine. These experimental results were in contrast with those obtained from spinal ganglion cells, where no appreciable change occurred in the potential height with variation of the external hydrazine concentration (Koketsu *et al.* 1959*b*).

Figure 4 shows the action potentials obtained in different concentrations of hydrazine. These records demonstrate that (1) the rate of rise of the action potential is apparently increased according to the increase of concentration and (2) the threshold for the initiation of the action potential and the negative after-potential increases as the external concentration decreases.

Effects of different concentrations of calcium ions

The resting potential of muscle fibres fell slightly with a decrease of the external calcium concentration and increased slightly in raised concentrations. The variation of the values observed in 80 mM hydrazine solutions containing 0·3–32 mM calcium ions was within ± 10 mV. When

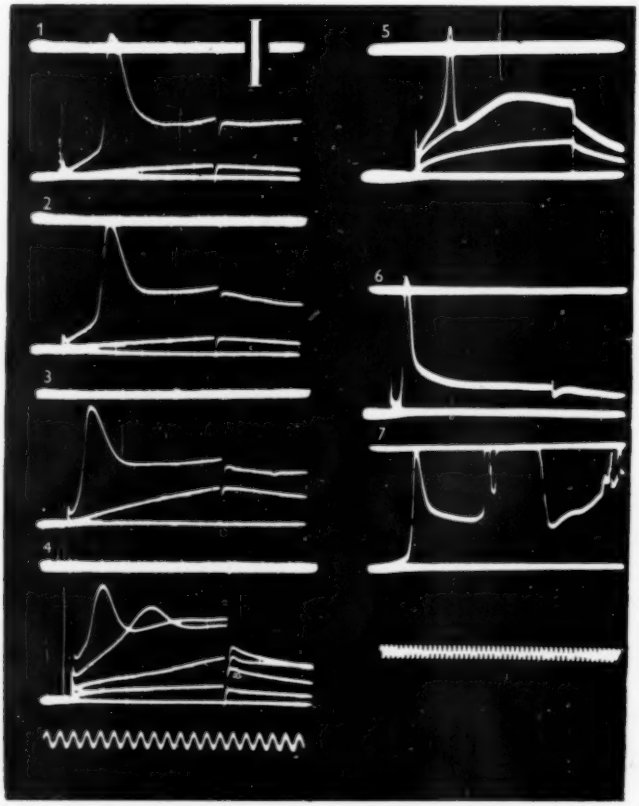

Fig. 4. Intracellular action potentials of muscle fibres soaked in solutions containing various concentrations of hydrazine. 1–4 are records contributing to Fig. 3, obtained in 112, 70, 50, and 30 mM hydrazine, respectively. Record 5 is obtained in hypertonic hydrazine solution (224 mM). Records 6 and 7 are conducted action potentials obtained in 224 and 90 mM hydrazine solutions, respectively (note rising phase, spike duration, and negative after-potential). The distance of recording and stimulating micro-electrodes inserted into a single fibre was approximately 0·5 (record 5), 1·0 (records 1–4), and 5 mm (records 6 and 7). Calibration, 40 mV, time marker, 1 msec.

the calcium concentration was reduced to 0·3 mM or increased to 9 mM, there was only a slight decrease of the threshold in lower concentrations and a slight increase in higher concentrations. Action potentials of the all-or-none type were no longer detectable when the calcium concentration was increased to 19 mM and most of the fibres then produced only small graded responses.

Effect of withdrawal of calcium ions

In frog spinal ganglion cells the excitability of cells was found to be eliminated when the calcium was completely withdrawn from the sodium-free chlorine, tetra-ethylammonium (TEA), or hydrazine solution. In the case of frog sartorius muscles the results were not as clear as those on spinal ganglion cells. When the muscle preparations, which were previously soaked in a sucrose solution for 30–120 min, were immersed in a Ca-free hydrazine solution, the value of the resting potentials of the individual fibres was found to vary. Some fibres had relatively high resting potential (70–80 mV), while other cells showed lower potentials (50–60 mV); the average value obtained from twenty-two fibres of a preparation was 71 mV. None of these fibres twitched spontaneously, which was generally observed in Ca-free Ringer's solution. The fibre having relatively high resting potentials (70–80 mV) produced action potentials, but many of them were of low or graded amplitude. The fibres with relatively low resting potentials (50–60 mV) did not produce action potentials, although some of these gave graded responses.

If the preparation was soaked previously in a calcium-free sucrose solution for 30–60 min, all fibres had low resting potentials (40–60 mV) and completely lost their electrical activity in the Ca-free hydrazine solution. The excitability of these preparations, however, could be restored upon reimmersion in the hydrazine solution containing Ca.

Intracellular injection

Muscle. Attempts to produce action potentials of muscle fibres by means of intracellular injections of hydrazinium ions were unsuccessful. Repeated injections of hydrazinium, as well as of TEA, did not induce any sign of restoration of the electrical activity in the sucrose, choline, or TEA solutions.

Spinal ganglion cell. The situation in spinal ganglion cells was quite different. Action potentials of cells soaked in sucrose solution were easily produced by intracellular injections of TEA (Koketsu *et al.* 1959*a*), hydrazine (Koketsu *et al.* 1959*b*), choline and acetylcholine. In every case local responses appeared initially and were followed by full spike potentials. The duration of these action potentials was gradually

prolonged by continuing the injections. Spontaneous activity, however, could not be observed even after repeated injections, indicating that these nitrogenous substances cannot activate the membrane without electrical stimuli.

It might be argued that the intracellularly injected substances might have diffused out across the membrane and in essence were acting extracellularly. In order to test this possibility, a KCl-filled electrode was inserted into a cell and hydrazinium ions were applied electrophoretically on the immediate outside of this particular cell by positioning a hydrazine-filled electrode close to the membrane surface. This procedure yielded negative results. Repeated intracellular injections of sodium ions did not restore the excitability of cells immersed in sucrose solution. It is also worth while to note that intracellular injections of hydrazine did not restore the excitability of cells which were immersed in a Ca-free sucrose solution.

After-effects of prolonged immersion

Muscle. Crustacean muscle fibres maintained their electrical activity in sodium-free solutions after previous immersion in a solution of quaternary ammonium ions (Fatt & Katz, 1953; Fatt & Ginsborg, 1958). This finding suggests that the ions which diffuse into the fibres during immersion are responsible for maintaining activity in sodium-free solutions. The same kind of experimental procedure was applied to the frog muscle to find whether it could maintain electrical activity in sodium-free sucrose solution after prolonged immersion in hydrazine solution. The electrical activity of the muscle was abolished within a minute when the preparations were placed in sucrose solution after prolonged previous immersion (up to 20 hr at 2–3° C) in a 112 mM hydrazine solution. The activity of these preparations was again restored within a minute by reimmersing in hydrazine solution, which suggests that the external hydrazine, rather than the internal, is important for the maintenance of electrical activity.

Spinal ganglion cells. The excitability of spinal ganglion cells could not be maintained for an extended time in 80–112 mM hydrazine solutions; the resting potential tended to drop gradually and many of the cells lost their excitability when they were soaked in these solutions for more than approximately 2 hr. This made it difficult to test the after-effect of an extended soaking. The excitability of cells was usually eliminated within 30–60 min in sucrose solution, but apparently maintained for a longer time than if they were previously soaked in a hydrazine solution for 1–2 hr (see Fig. 5).

Such an after-effect was more clearly demonstrated by using TEA solution. If the spinal ganglia were previously immersed in TEA

solution for a certain period (4–20 hr.), the excitability of cells was well maintained and produced potentials for several hours in sucrose solution (see Fig. 5).

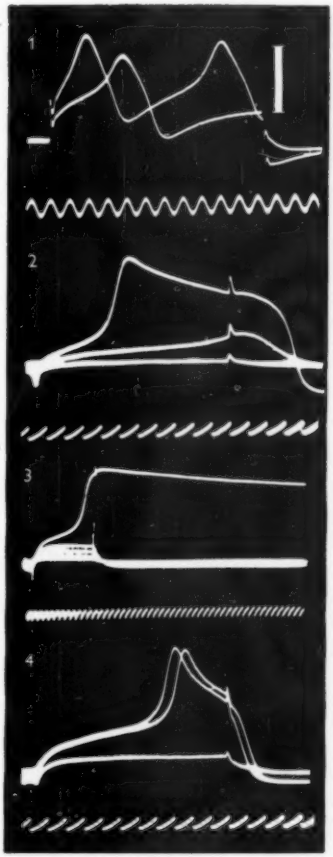

Fig. 5. Intracellular action potentials of spinal ganglion cells soaked in sucrose solution after previous immersion in hydrazine (1) and TEA (2–4) solutions. Record 1 obtained 90 min after soaking in sucrose (2 hr previous immersion in 112 mM hydrazine at room temperature). Record 2 obtained 2·5 hr after soaking in sucrose (4 hr previous immersion in 112 mM TEA at room temperature). Records 3 and 4 obtained 30 min and 5 hr after soaking in sucrose (20 hr previous immersion in 112 mM TEA at 2° C). Calibration, 40 mV, time marker, 1 msec.

DISCUSSION

According to the ionic hypothesis (Hodgkin, 1951), the action potential of nerve impulses is generated by the influx of the sodium ions which shifts the resting membrane potential toward the sodium equilibrium potential. It must be that trace amounts of sodium ions are retained in the intracellular space or immediately outside the membrane, even when the preparations are soaked in sodium-free solutions for an extended time. Thus, it might be argued that these sodium ions are still responsible for the production of action potentials in sodium-free media. This possibility, however, seems to be inconceivable, since, first, the action potentials produced in hydrazine solutions are too large to be explained in this way, secondly, the electrical activity of isolated single muscle fibres is maintained indefinitely in hydrazine solution, and finally, the electrical activity of the muscle is easily eliminated in sodium-free sucrose or choline solutions.

If the excitatory process in hydrazine solution is essentially identical with that in normal Ringer's solution, it would be expected that the hydrazinium ions are acting exactly in the same manner as sodium ions. A significant relationship between the concentration of the external sodium ions and the spike height of the action potential has been found in various excitable tissues, which, supports the sodium hypothesis (Hodgkin & Katz, 1949; Nastuk & Hodgkin, 1950; Huxley & Stampfli, 1951). A similar result was observed in muscle fibres immersed in hydrazine solution, although the spike height in hydrazine was smaller than that in sodium. Comparable results were obtained from frog nerve axons soaked in a guanidinium solution (Lüttgau, 1958). It may be possible to introduce a hypothesis involving such factors as the equilibrium potential, the permeability constant or even a pumping mechanism of hydrazinium ions to explain the role of hydrazine in the maintenance of membrane excitability. It also seems feasible, however, to assume that hydrazinium ions are acting in a manner which cannot be explained by the sodium hypothesis.

Entrance of hydrazinium ions could not be held responsible for active depolarization if intracellular injections of hydrazinium ions are necessary to produce action potentials in sucrose solution. In ganglion cells intracellular hydrazine appears to be essential for the restoration of electrical activity. The possibility that hydrazinium ions injected into the cell body diffuse out and act extracellularly may be eliminated for the following three reasons: (1) an external hydrazine concentration less than 80 mM could not produce full-spike potentials (Koketsu *et al.* 1959*b*); (2) external application of hydrazinium ions immediately outside the membrane did not produce action potentials; and (3) action potentials were not produced

by intracellular sodium injections in sucrose solution. No explanation is available at this moment why no action potentials were produced by intracellular injections of hydrazinium ions into the muscle fibres. The results of the present experiments rather suggest that the external hydrazinium ions are essential for the production of action potentials. Two possibilities may be considered: (1) hydrazinium ions are acting as charge carriers, probably like sodium ions, in the muscle but not in spinal ganglion cells; and (2) hydrazinium ions can exert their specific effect externally on the muscle membrane although internally on the cell membrane.

There seems to be a reduction of the membrane resistance during the action potential in sodium-free solutions (Koketsu *et al.* 1959*a*). If the increase of conductance is due to an increased permeability of the membrane to certain kinds of ions, the active depolarization may be explained either by an increased influx of external cations or an efflux of internal anions. Calcium ions are among the external cations which must be considered in this case. Fatt & Ginsborg (1958) attempted to explain the electrical activity of muscle fibres of crayfish in sodium-free media on the basis that the substituted ammonium compounds increased the permeability of the active membrane to calcium ions. In the present experiment, however, no appreciable relationship was found between the amplitude, duration or maximum rate of rise of the action potentials and the calcium concentration in the hydrazine solution, suggesting that calcium is not acting as charge carrier for the generation of action potentials. A similar result was also observed in the nerve axon in sodium-free guanidinium solution (Lüttgau, 1958). The presence of calcium ions, however, seems to be indispensable for the restoration of electrical activity in muscle fibres as well as in spinal ganglion cells (Koketsu *et al.* 1959*b*) in sodium-free solutions. The importance of external calcium for the electrical activity in sodium-containing solutions has been reported for the squid giant axon (Frankenhauser & Hodgkin, 1957) and frog motor axon (Frankenhauser, 1957), but the mechanism of calcium action in the excitatory process is still obscure.

An important question which remains is whether the excitatory process in sodium-free solutions is essentially identical with that in normal Ringer's solution. It is conceivable that the various nitrogenous compounds substituted for sodium exert some specific action on the tissues and produce action potentials whose mechanism is fundamentally different from the normal excitatory process.

SUMMARY

1. The electrical activity of isolated single muscle fibres can be maintained in a sodium-free hydrazine solution.

2. External chloride ions can be replaced by nitrate or sulphate ions for maintaining excitability of muscle fibres in a sodium-free hydrazine solution.

3. Calcium ions are essential for maintaining excitability in a sodium-free hydrazine solution. They did not seem, however, to be acting as charge carriers during active membrane depolarization.

4. The relationship between the active membrane potential and the external hydrazine concentration indicates that a reduction to one-tenth of the external hydrazine concentration results in approximately 58 mV decrease of the spike height.

5. Intracellular injections of hydrazine into the muscle fibres did not restore excitability. There was, however, strong experimental evidence indicating that the intracellularly injected hydrazine was responsible for maintaining the excitability of spinal ganglion cells.

6. No experimental evidence for an essential role of intracellular hydrazine was found in muscles. Results rather suggest that external application of hydrazine is important for the maintenance of muscle excitability.

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PENETRATION OF BROMOPHENOL BLUE FROM THE PERFUSED CEREBRAL VENTRICLES INTO THE BRAIN TISSUE

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In the present experiments the uptake of a substance from the inside of the brain was studied by perfusing the cerebral ventricles with the dye bromophenol blue. Depth and intensity of its penetration into the brain tissue were observed with the naked eye, thus allowing a detailed study of the topography of its uptake.

In recent experiments on anaesthetized cats it was found that histamine perfused through the cerebral ventricles penetrated into the brain tissue surrounding the ventricles (Draškoci, Feldberg, Fleischhauer & Haranath, 1960). This uptake of histamine from the inside of the brain was not uniform. When samples of tissue removed from different parts of the ventricular wall were extracted and assayed for histamine, it was found that those parts of the wall which are formed by grey matter had taken up more histamine than those parts which are formed by white matter. There were also differences in uptake between different regions of grey matter. Similar observations were made by Roth, Schoolar & Barlow (1959) with labelled acetazoleamide (Diamox) injected intravenously into cats. This inhibitor of carbonic anhydrase was found to be first secreted into the ventricular fluid, from which it passed more deeply into grey than into white matter bordering the ventricular cavities.

The results obtained with histamine and acetazoleamide are at variance with the current views about the passage of substances from the c.s.f. into the brain tissue, which is generally regarded as a diffuse process independent of structural differences of the brain. These views originate from Goldmann's (1913) classical experiments with the acidic dye trypan blue which has also been used in much of the later work on the problem. Trypan blue penetrates very slowly into the brain tissue and there are no differences of penetration between different regions of the brain. A number of other acidic dyes as well as potassium ferrocyanide were found to behave

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similarly. Spatz (1933) therefore concluded that with regard to the uptake of substances from the cerebrospinal fluid the brain behaves like a colloidal mass of homogeneous structure. Bakay (1956) arrived at somewhat similar conclusions from experiments on the penetration of ^{32}P into brain tissue.

In order to re-examine the problem we looked for a dye which would not pass the blood-brain barrier, but which would penetrate the brain tissue better than trypan blue. The acidic dye bromophenol blue seemed to fulfil these conditions. Millen & Hess (1958) showed that it did not pass the intact blood-brain barrier when injected intraperitoneally, but that at sites where the barrier was damaged it penetrated much faster and deeper into the brain tissue than did trypan blue.

METHODS

The experiments were carried out on 10–12 month-old cats, weighing between 2·1 and 2·8 kg. They were anaesthetized by an intraperitoneal injection of pentobarbitone sodium 35 mg/kg. The treachea was cannulated and loose threads were placed round the exposed carotid arteries to facilitate their cannulation at the end of the experiment.

The methods of perfusion of the cerebral ventricles from the lateral ventricle to the aqueduct, so as to exclude the fourth ventricle and the subarachnoidal space, are described elsewhere (Draškoci *et al.* 1960). Perfusion with a solution containing the dye bromophenol blue was begun after the ventricles had been perfused for 10–30 min with an artificial salt solution of a composition resembling that of cerebrospinal fluid. This solution, which was introduced by Merlis (1940), will be referred to as artificial c.s.f. Its composition is as follows: (g/l.) NaCl 8·1; KCl 0·25; $CaCl_2$ 0·14; $MgCl_2$ 0·11; $NaHCO_3$ 1·76; NaH_2PO_4 0·07; $(NH_2)_2CO$ 0·13, and glucose 0·61. For the dye perfusion the sodium salt of bromophenol blue was added to this fluid. Unless otherwise stated, the rate of perfusion was 0·1 ml./min. In preliminary experiments it was shown that the sodium salt of bromophenol blue injected intravenously into cats did not pass the blood-brain barrier.

Bromophenol blue was obtained commercially (British Drug Houses, Ltd) as the acid, in a powder form which is insoluble in water. Its molecular weight is 669·9. The water-soluble sodium salt was prepared by grinding the powder in a mortar with equimolecular amounts of N/10-NaOH, i.e. about 6 ml. for each 400 mg powder. The sodium salt of bromophenol blue has a blue-violet colour. It changed the pH of the artificial c.s.f. from about 8·6 to between 8·2 and 7·8 when added to it in a concentration of 0·1–0·2 %. These concentrations, which were used for perfusion of the cerebral ventricles, refer to the concentration of acid and not of the sodium salt. Both the solution of the sodium salt of bromophenol blue and the artificial c.s.f. were freshly prepared for each experiment.

At the end of the perfusion the dye solution in the ventricular cavities was washed out by a short perfusion with artificial c.s.f. The head was then perfused from a cannulated carotid artery with saline solution followed by a solution of 10 % formaldehyde. The fixation with formaldehyde had no influence on the depth and distribution of the staining. Unfixed brains, removed and investigated quickly after killing the animal by an overdose of pentobarbitone sodium, showed the same pattern of staining.

Immediately after the formalin perfusion of the head the brain was removed, frozen on a freezing microtome and cut either in a horizontal or in a nearly frontal plane. The frontal plane corresponded as closely as possible to the plane in Winkler & Potter's (1914) atlas of the cat's brain. In order to obtain permanent records of the dye penetration at different levels of the brain, a series of photographs was taken of the surface of the frozen brain at different

stages of the sectioning. The freezing and photographing of the brain was always carried out immediately after its removal in order to prevent any post-mortem diffusion, because when the brain was kept unfrozen in formalin a gradual diffusion of the dye into the surrounding fluid took place. Such diffusion could be reduced to a great extent, but not prevented, by keeping the unfrozen brain in liquid paraffin.

RESULTS

When bromophenol blue was perfused from the left lateral ventricle to the aqueduct with the free end of the outflow cannula about 1 cm below the aqueduct, the right lateral ventricle was often less well perfused than the left; so that the wall of the left lateral ventricle became more intensely stained than that of the right ventricle, which was sometimes not stained at all. This uneven perfusion of the two lateral ventricles is attributed to the slight suction exerted by the low outflow level, which prevented the perfusion fluid from entering the right lateral ventricle on its passage via the third ventricle to the aqueduct. The uneven perfusion could be greatly reduced and sometimes prevented when the free end of the outflow cannula was kept only a few millimetres below the aqueduct, but with this arrangement there was the danger of leakage of small amounts of dye along the outside wall of the cannula. Such leakage of dye invariably occurred when the end of the aqueductal cannula was raised to the level of the aqueduct; in these experiments the membrane which forms the thin roof of the posterior end of the third ventricle sometimes became damaged or ruptured, so that larger amounts of dye appeared in the subarachnoidal space.

In most perfusion experiments the free end of the outflow cannula was at a level about 1 cm below the aqueduct and the penetration of the dye was examined in the walls of the third and left lateral ventricles only. In a few experiments both lateral ventricles were cannulated and alternately perfused for periods of 20 min each.

When a 0·2 % solution of bromophenol blue was perfused through the cerebral cavities for 2 hr, the dye penetrated so deeply from the inside into the brain substance that some areas of the outside became stained. This staining was more intense when perfusion was maintained for 5 hr with a 0·1 % solution of the dye. Such an experiment is illustrated in Pl. 1.

Deep staining has occurred at the outside of the brain in four regions in which the ventricular cavities are separated from the surface by a relatively thin mass of nervous tissue: (1) The floor of the third ventricle is intensely stained, but the hypophysis has remained unstained (Pl. 1*A*). (2) The mammillary bodies are well stained, although not as intensely as the floor of the third ventricle; in picture *A*, the mammillary bodies cannot be seen as they are hidden under the hypophysis, but their staining is seen in the sagittal section at *C*. (3) Parts of the temporal lobe are well stained (Pl. 1*A*), because the dye has penetrated from the inferior horns

of the lateral ventricles to the outside of the brain. (4) Practically the whole surface of the olfactory lobe is intensely stained by the penetration of the dye from the olfactory recesses of the lateral ventricles (Pl. 1*B*, *C*). In addition, the septum pellucidum, the medial wall of the lateral ventricle, is intensely stained, as is seen when the brain is cut in the mid line (Pl. 1*C*).

Apart from these regions no dye has reached the outside surface of the brain. As is shown in Pl. 1, large areas of the ventral and medial surface and the whole lateral surfaces of the cerebral hemispheres are unstained. In these regions the surface of the brain is separated from the ventricular cavities by thick masses of white matter.

The median section of the brain, shown in Pl. 1*C*, illustrates some additional features of staining from the third ventricle and aqueduct. (1) The wall of the third ventricle is intensely stained and the dye has filled the cavity of the infundibulum, but it has not penetrated into the tissue of the hypophysis. (2) The upper parts of the optic chiasma which form the anterior part of the floor of the third ventricle are slightly stained. (3) The dye has penetrated from all sides into the massa intermedia, which is a relatively large structure in cats; its centre stands out in the photograph as a light grey spot. In the freshly cut brain this region showed a light bluish colour which proved that some dye had penetrated throughout the massa, although its periphery was more intensely stained. (4) In contrast to the hypophysis the pineal body is intensely stained; it was elevated by placing a small piece of white paper behind it before taking the photograph. (5) The central grey matter around the aqueduct is well stained. This is shown in the anterior part of the mesencephalon, where the section passes slightly lateral to the lumen of the aqueduct. In the posterior part of the mesencephalon the section went through the lumen of the aqueduct, but the dark colour here is due to a blood clot which had formed at the tip of the cannula. There was no staining of the floor and walls of the fourth ventricle; the black area seen below the posterior part of the cerebellum is due to a blood clot.

Plate 2 shows the penetration of the dye from the ventricular cavities in four horizontal sections, the levels of which are indicated in Pl. 1*C*. In this experiment perfusion was carried out with 0·2% bromophenol blue for 90 min at a rate of 0·2 ml./min. Each section is accompanied by a drawing, as the photographs do not bring out the shapes of the ventricular cavities. They were, however, discernible by slight contrast in the negative and could therefore be drawn when the negatives were projected on a larger screen. Use is made of these drawings in connexion with the photographs when describing the penetration of dye as seen in the four sections.

Section 1 illustrates the intense staining of the olfactory bulb from the olfactory recesses of the lateral ventricles. The difference between the two

sides is due to the fact that the section was not cut exactly in the horizontal plane.

From section 2 the following facts emerge: (1) The medial parts of the caudate nuclei are intensely stained. The dye has penetrated for about 2·5 mm into the nuclei; only their lateral parts are unstained. (2) The fibres of the corpus callosum are stained but penetration is not as deep as into the caudate nuclei and the staining is less intense. The photograph shows clearly that the dye had not penetrated the whole thickness of the white matter of the corpus callosum, although it forms a layer of tissue thinner than the layer of the caudate nucleus which had been penetrated by the dye. (3) The septum pellucidum is intensely stained. The light greyish spot in its middle is formed by unstained or slightly stained fibres of the fornix. (4) The section passes through the massa intermedia which is intensely stained except in its centre. Here the tissue is only faintly stained. (5) The walls of the third ventricle anterior and posterior to the massa intermedia are intensely stained and in the anterior parts the staining of the thalamus is continuous with that of the caudate nucleus. (6) The walls of the inferior horns of the lateral ventricles are stained. The staining is more pronounced and penetration is deeper in the medial walls formed by the hippocampus than in the lateral wall, which consists mainly of white matter. These details were clearly seen in the fresh brain and are shown in the drawing. They are not brought out in the print. Photographic evidence, however, for the differential staining of the walls of the lateral ventricle is given in Pl. 3, figs. 1 and 2.

Section 3 is cut a few millimetres more dorsal than section 2; it passes through the third ventricle above the massa intermedia in the region of the epithalamus, then through the posterior commissure and finally through the anterior part of the aqueduct. The staining of the walls of the lateral ventricles is similar to that described for section 2, but the staining from the third ventricle and the aqueduct shows in addition that the whole of the central grey matter is stained intensely, as are the walls of the third ventricle with the exception of its posterior part, which is formed by the posterior commissure. Here the staining is less intense. Penetration into the wall of the third ventricle is not quite as deep as into the tissue of the caudate nucleus. This difference was clearly seen in several, but not in all experiments.

Section 4 is cut dorsal to the third ventricle and passes through the central parts of the lateral ventricles into which the right and left hippocampus protrude as large ovoid structures. The difference in their shape results from the fact that the section is slightly more dorsal on the left than on the right side. The section illustrates the intense staining of the superficial parts of the hippocampus, which consist of the thin alveus, the

dendrites of the lamina zonalis and the cellular layer of Ammon's horn. In comparison, those parts of the ventricular wall which consist predominantly of myelinated fibres are stained less intensely.

Plate 3, fig. 1 shows the penetration of dye in two frontal sections after perfusion of the cerebral cavities for 2 hr with 0·2 % bromophenol blue at a rate of 0·1 ml./min. The plane of section *A* lies a little posterior to that of *B* and passes through the distal ends of the pineal body and of the neural lobe of the hypophysis, whereas section *B* passes through the middle of these structures. Both sections show the intense staining of the pineal body and the absence of any stain in the neurohypophysis. Both sections show a sharp demarcation between the grey matter surrounding the aqueduct and the white matter. The central grey matter is stained intensely throughout its whole width; it is surrounded by a faintly stained zone of white matter which includes the posterior commissure. The staining of the medial wall of the lateral ventricles differs in the two sections, being continuous in *A* and interrupted in *B*. In section *A* the medial wall is formed entirely by the hippocampus and the whole wall is stained. In the more anterior section *B* part of the medial wall is formed by the white matter of the fimbria which emerges from the hippocampus. The fimbria is unstained; therefore the staining of the medial wall is interrupted. The staining of the lateral walls of the lateral ventricles shows that the dye has penetrated everywhere into the brain tissue, but that the penetration is deeper and more intense in the floor of the inferior horn where the wall is formed by the grey matter of the pyriform lobe.

Plate 3, fig. 2, is the lower part of a frontal section from another experiment in which the cerebral cavities were also perfused for 2 hr at a rate of 0·1 ml./min. In this experiment the dye has not entered the right lateral ventricle, but the staining from the left was more pronounced than in the experiment of fig. 1. From the floor of the left inferior horn the dye has penetrated through nearly the whole wall of the pyriform lobe and the hippocampus. Such deep penetration throughout almost the whole thickness of the pyriform lobe accounts for the outside staining of the temporal lobe, as is illustrated in Pl. 1. The plane of the section shown in Pl. 3, fig. 2 is slightly anterior to that of section *B* in Pl. 3, fig. 1; it passes through the mammillary bodies which are intensely stained. The cavity of the infundibulum below the mammillary bodies is filled with the dye and its walls are stained, but no dye has passed into the anterior lobe of the hypophysis, which is cut below the infundibulum.

In a few experiments the cerebral cavities were perfused with 0·2 % bromophenol blue for 30 min to 1 hr. These shorter periods of perfusion were sufficient to allow the dye to penetrate deeply into the brain substance, but the intensity of the staining was less than in the experiments in which

perfusion was continued for longer periods. Plate 3, fig. 3 illustrates the staining obtained after perfusion during 30 min. The dye has penetrated the pineal body and the central grey matter which is stained throughout and the penetration here is already as intense as in the experiment of fig. 1 after perfusion for 2 hr, but the surrounding zone of faintly stained white matter is practically absent. The section passes through both lobes of the hypophysis. The anterior and the posterior lobes are unstained, although the infundibular cavity is filled with dye.

No penetration of dye into the brain substance was seen when the perfusion was started a few minutes after killing the cat by an overdose of pentobarbitone sodium. This is illustrated by the experiment of Pl. 3, fig. 4, in which the cerebral ventricles were perfused with a 0·2% bromophenol blue solution, started 4 min after a lethal intravenous injection of pentobarbitone and continued for 2 hr. In the freshly cut brain the whole surface area of the ventricular cavities was blue, but the dye had not penetrated into the brain tissue.

DISCUSSION

The present experiments show that bromophenol blue, when perfused through the cerebral ventricles of an anaesthetized cat for ½ hr or longer, passes relatively deeply into the nervous tissue. This finding is in accord with the observations of Millen & Hess (1958) that, at sites where the blood-brain barrier was damaged, intraperitoneally injected bromophenol blue penetrates more readily into the brain substance than trypan blue.

The passage of bromophenol blue is not merely a process of diffusion. The absence of penetration in experiments in which perfusion was started a few minutes after the cat was killed shows that vital processes are involved in the uptake of the dye.

The penetration of bromophenol blue into the ventricular wall was found to vary in different regions, depending on structural differences of the wall and, like the results previously obtained with histamine and labelled acetazoleamide, does not agree with the idea held by Spatz (1933) and Bakay (1956) of a diffuse penetration into the brain tissue independent of its structural differences.

With histamine it had been found that more is taken up by grey matter than by white, and more by the caudate nucleus than by the grey matter of the wall of the third ventricle (Draškoci *et al.* 1960). With acetazoleamide penetration from the ventricular cavities was most pronounced in the caudate nucleus, hypothalamus and portions of the hippocampus (Roth *et al.* 1959). The penetration of bromophenol blue into the ventricular wall showed a somewhat similar pattern and many of the findings

can be accounted for by a more ready penetration into grey than into white matter.

From the lateral ventricle the dye penetrated deeply into the grey tissue of the caudate nucleus, through the whole wall of the septum pellucidum and, from the inferior horn, through practically the whole pyriform lobe. There was also deep penetration into the hippocampus but not as deep as into the tissue of the caudate nucleus. On the other hand, the dye penetrated for a much shorter distance into those parts of the wall which are formed by white matter, such as the corpus callosum, the fimbria and the whole lateral wall; in addition, the staining of the penetrated white matter was also less intense. On the assumption that the dye passes more readily into grey than into white matter, it is possible to explain why penetration into the hippocampus is less intense than into the caudate nucleus: the grey tissue of the hippocampus is covered by the alveus, a thin layer of white matter.

From the third ventricle the dye penetrated deeply into the grey tissue of the hypothalamus and of the massa intermedia, whereas penetration into the white tissue of the posterior commissure and of the chiasma was again comparatively slight. The finding that in several experiments penetration into the tissue of the hypothalamus was not as deep as into the tissue of the caudate nucleus is in accord with the previous experiments on the uptake of histamine. In these it was found that the caudate nucleus had usually taken up more histamine than the hypothalamus. The differences may reflect the fact that a large number of myelinated nerve fibres is found in the wall of the third ventricle at a relatively short distance from the ventricular surface so that the grey matter forms a thinner layer of tissue than the large mass of the caudate nucleus.

From that part of the aqueduct which was included in the perfusion, the dye penetrated through the whole width of the central grey matter even when perfusion was continued for 30 min only; with longer periods of perfusion the dye penetrated also into the white matter, which became faintly stained, but the sharp demarcation between the two structures remained conspicuous, emphasizing the difference of penetration between grey and white matter.

Both the hypophysis and the pineal body are structures in which the blood–brain barrier is relatively permeable (Wislocki & Leduc, 1952). It was therefore surprising that they behaved differently with regard to the uptake of bromophenol blue from the third ventricle, as was evidenced by the intense staining of the whole pineal body and the absence of penetration of dye into the tissue of the hypophysis.

The absence of any staining in the anterior lobe of the hypophysis is understandable, since this glandular tissue is connected with the brain

by the portal vessels only and is nowhere in direct contact with the ventricular fluid. On the other hand, the posterior lobe which consists of many unmyelinated nerve fibres and specialized glial cells, the pituicytes, is in close contact with the ventricular fluid through the cavity of the infundibulum, which in cats extends deeply into this part of the hypophysis. Here, therefore, the barrier between c.s.f. and brain must have special properties. The barrier is somewhat similar to that at regions of white matter, but with this difference: at the neurohypophysis the barrier is impermeable to bromophenol blue, whereas at regions of white matter some penetration of dye occurs.

The deep staining of the pineal body suggests a resemblance to the barrier at regions of grey matter. Yet the analogy may be misleading, since the pineal body consists mainly of neuroglial and parenchymal cells, which are not nerve cells, although they are both derived from the neuroectoderm.

One factor which may influence the penetration of bromophenol blue into the brain tissue is the structure of the ependyma, a layer of specialized neuroglial cells which covers the whole surface of the cerebral ventricles, including that of the hypophysis and pineal body. It is unlikely that substances taken up from the ventricular fluid can pass intercellularly between the ependymal cells, because the extracellular space between adjacent cells is only about 200 Å wide. In addition, the apical parts of the cell membranes are thickened by terminal bars and there is elaborate indentation and overlapping between adjacent cells (Fleischhauer, 1957). The passage of substances is therefore most likely transcellular, which accords with the view that the passage is an active process.

It is reasonable to assume that regional differences in the cytology of the ependymal cells affect the penetration of the dye. Such differences have been shown to exist. An extensive study of the ependyma of the turtle's brain revealed that each region of the ventricular wall is characterized by a special structure of its ependyma (Fleischhauer, 1957), and characteristic regional patterns have also been found in the ependymal structure of the ventricular walls of the cat (Fleischhauer, 1958, 1960).

Regional differences in the cytology of the ependymal cells cannot be solely responsible for the observed differences of penetration. For instance, the sharp demarcation between the deep staining of the central grey matter and the faint staining of the surrounding white matter suggests that differences of penetration within the brain tissue are, to some extent at least, determined by inherent properties of the grey and white matter. The known cytological differences between the neuroglia of grey and white matter may be responsible for these differences. On this assumption the active process which allows the passage of dye through the ependyma as

well as into the brain tissue would essentially be a function of different forms of neuroglial cells.

The present experiments are confined to bromophenol blue, but since the uptake of histamine and of acetazoleamide appears to be similar, a common pattern emerges for the penetration of substances from the ventricular cavities into the ventricular walls. The fact that these different substances penetrate relatively large distances of grey matter and that bromophenol blue crosses the whole wall in some regions of the brain, brings to mind a favourite idea of the Swiss neurologist von Monakow (1921), who believed that the c.s.f. may pass from the ventricles through the whole mass of the brain and, on its way, convey a number of hormonal substances to nuclear regions. Von Monakow's views find support in the comparative histology of the cerebral ventricles and particularly in the fact that in lower vertebrates long processes originate from the ependymal cells and pass perpendicularly throughout the whole wall of the brain, sending off numerous branchings before terminating in the external limiting membrane. The results of the present experiments with bromophenol blue are in accord with von Monakow's views, at least in so far as they show that a relatively large molecule, which does not pass from the blood stream into the brain tissue, can penetrate deeply into nuclear regions from the ventricular cavities. This mode of entry into the brain tissue is of physiological and pharmacological interest, particularly for substances which, like ^{32}P (Bakay, 1956) and acetazoleamide (Roth *et al.* 1959), pass the blood–brain barrier very slowly, but are quickly secreted into the ventricular fluid.

SUMMARY

In anaesthetized cats the cerebral ventricles were perfused from a cannulated lateral ventricle to the middle of the aqueduct with a 0·1 or 0·2% solution of the acidic dye bromophenol blue. Its penetration into the ventricular walls after perfusion for 30 min or longer was studied with the naked eye in the whole brain and during sectioning of the formalin-fixed frozen brain.

1. Deep penetration and intense staining occurs in those regions of the ventricular wall which consist mainly of grey matter, such as the caudate nucleus, septum pellucidum, pyriform lobe, hippocampus, hypothalamus and the central grey matter.

2. In contrast, the dye penetrates a short distance only, and staining is less intense, in those regions of the ventricular walls which consist of white matter, such as the lateral walls of the lateral ventricles, the corpus callosum, the optic chiasma and the posterior commissure.

3. In regions in which the surface of the brain is separated from the ventricular cavities by a relatively thin mass of nervous tissue consisting mainly of grey matter, the dye penetrates to the outside of the brain and stains its surface. These regions are the floor of the third ventricle, the mammillary bodies and parts of the temporal lobe.

4. The hypophysis and the pineal body behave differently with regard to the uptake of bromophenol blue from the third ventricle. No dye penetrates into the tissue of the anterior and posterior lobes of the hypophysis, whereas the pineal body is intensely stained.

5. The uptake of bromophenol blue by brain tissue is an active process and does not occur when perfusion of the cerebral ventricles with the dye is carried out after killing the cat.

6. In the discussion it is suggested that the differences in dye uptake by the various regions of the brain may result from differences in their neuroglia.

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[OVER

EXPLANATION OF PLATES

PLATE 1

Appearance of cat's brain after 5 hr perfusion of the lateral and third ventricles with 0·1 % bromophenol blue. *A*, ventral surface; *B*, lateral surface; *C*, medial surface. The brain is cut near the mid line and a small piece of white paper is placed behind the pineal body. The planes 1, 2, 3 and 4, refer to the horizontal sections shown in Pl. 2.

PLATE 2

Four horizontal sections of cat's brain after 2 hr perfusion of the lateral and third ventricles with 0·2 % bromophenol blue. The planes of the sections are indicated in Pl. 1*C*. The drawings on the right were made from the negatives to show the outlines of the ventricles and the depth of dye penetration. The black spot in the left frontal lobe of section 4 is made by the blood-stained track of the intraventricular cannula. AQ, aqueduct; CC, corpus callosum; CN caudate nucleus; Fi, fimbria; Hi, hippocampus; LV lateral ventricle; MI, massa intermedia; PC, posterior commissure; OR, olfactory recess of lateral ventricle; SP, septum pellucidum; III, third ventricle.

PLATE 3

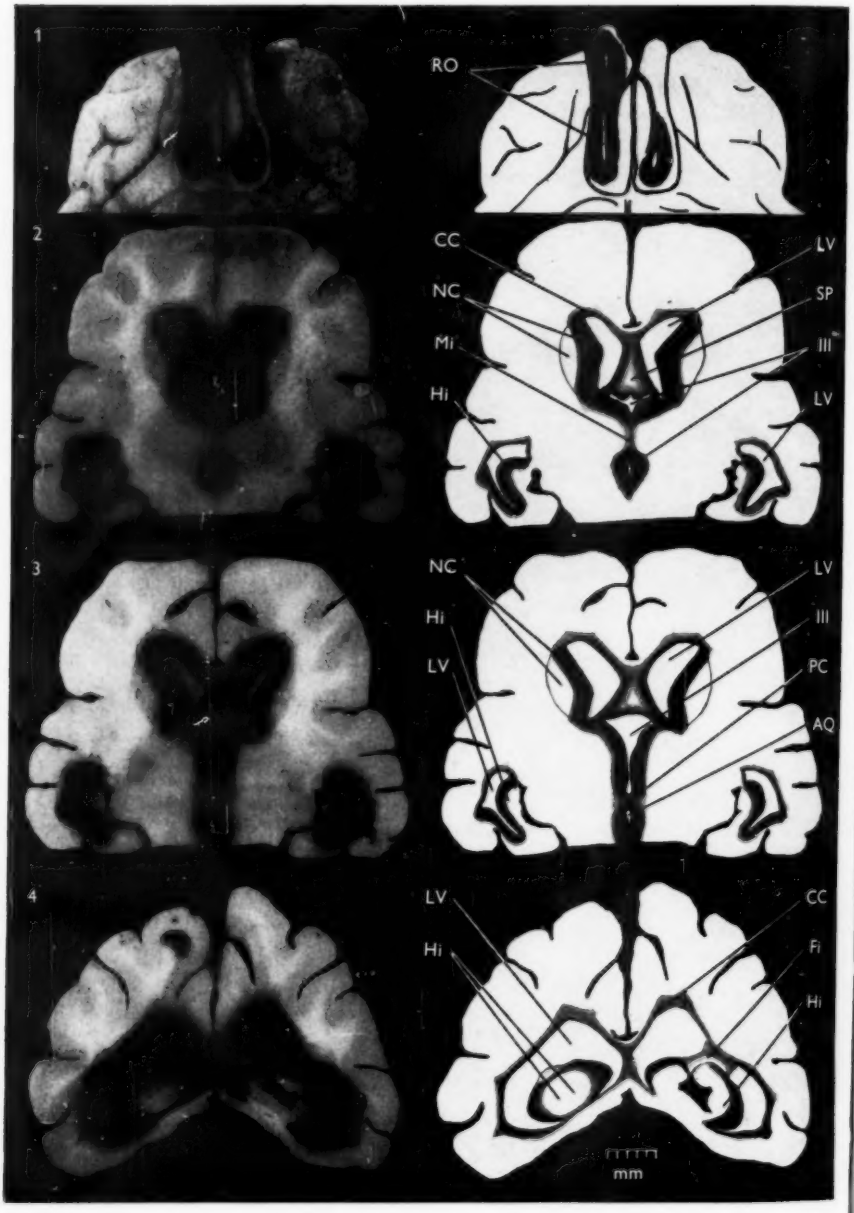
Fig. 1. Two coronal sections (*A* more posterior than *B*) of cat's brain after 2 hr. perfusion of the lateral and third ventricles with 0·2 % bromophenol blue. The arrow in *B* points to the fimbria.

Fig. 2. Lower part of a coronal section of cat's brain after 2 hr perfusion of the lateral and third ventricles with 0·2 % bromophenol blue. No dye had entered the right lateral ventricle. P, pyriform lobe.

Fig. 3. Coronal section of left half of cat's brain after 30 min perfusion of the lateral and third ventricles with 0·2 % bromophenol blue.

Fig. 4. Coronal section of cat's brain after 2 hr perfusion of the lateral and third ventricles with 0·2 % bromophenol blue. Perfusion was started 4 min after a lethal intravenous injection of pentobarbitone sodium.

A
B
C
4
3
2
1



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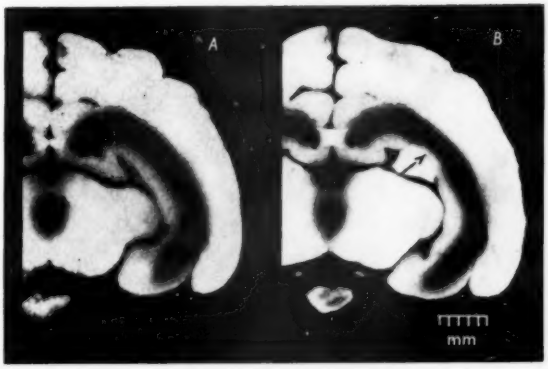

Fig. 1

Fig. 2

Fig. 3

Fig. 4

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REFLEX ACTIVATION OF INTERCOSTAL NERVES AND TRUNK MUSCLES BY NON-MYELINATED FIBRES OF THE SPLANCHNIC NERVE IN RABBITS

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Kugelberg (1948) has reported a double reflex discharge into leg flexors as a feature of the pathological flexion reflex in man. Using differential blocking agents, such as asphyxia or procaine upon the sensory nerve, he produced evidence that the early and the late responses of muscle could be related to afferent volleys in A and C fibres, respectively. Conduction velocity of the slow afferent volley was of the order of 1 m/sec. Ashby (1949) also has reported cases in which a delayed withdrawal reflex of the legs is associated with the delayed pain sensation. The delay in the two responses indicated that the afferent volleys were in fibres of slow conduction.

Earlier workers have reported that afferent C fibres can evoke skeletal muscle reflexes as well as other responses in experimental animals. Ranson & Billingsley (1916) showed that cutting the lateral division of the dorsal root, carrying the non-myelinated fibres, in cats, abolished the changes in B.P. and breathing and the struggling which resulted from dorsal root stimulation. Clark, Hughes & Gasser (1935) correlated reflex changes in breathing and B.P. with C-fibre activity in the cat saphenous nerve which continued after blocking myelinated fibres by compression or asphyxia. Bishop & Heinbecker (1935*a*) also found that non-myelinated afferent fibres could evoke breathing, B.P. and pupil responses and also skeletal muscle movements in cats, dogs and rabbits. These responses were recorded after blocking all but the C fibres with a burst of faradic shocks to the sensory nerve, and were seen most regularly in animals under Dial (allobarbitone; Ciba) anaesthesia. In their one experiment using the splanchnic nerve as the afferent pathway in a dog they failed to elicit any responses. There are many reports of skeletal muscle reflexes set up by afferent volleys in the splanchnic nerves (see Downman, 1955) and these have usually been related only to the activation of small myelinated fibres. Tower (1933) noted that the slower afferent impulses in sympathetic nerves evoked limb movements in decerebrated frogs, but could not relate these movements to any particular fibre group.

In this paper an account is given of a double reflex discharge into skeletal muscle in response to an afferent splanchnic volley. Evidence is presented that the second reflex discharge is set up by the slowly conducted volley in the non-myelinated afferent fibres.

METHODS

Adult rabbits were anaesthetized or decerebrated. Anaesthesia was induced with Dial (allobarbitone; Ciba) 1·0 ml./kg body wt. I.V., and maintained with small repeated doses. For decerebration anaesthesia was induced either with ether inhalation, or with thiopentone sodium (Intraval; May & Baker) 1·0 ml. of 5% solution intravenously. After tying both common carotid arteries, the brain above the intercollicular level was removed by suction through a trephine opening in the skull. Bleeding was reduced by injecting hexamethonium iodide (Hexathide; Allen & Hanbury) 1·0 mg intravenously before opening the skull, coupled with pressure on the vertebral arteries under the wings of the atlas if needed. The basilar artery or circle of Willis was clipped as it was drawn up into the sucker. In all cases the trachea was cannulated as soon as the animal was anesthetized.

Intercostal nerves, one or more of the 9th to 12th of the left side, were dissected clear from between the intercostal muscles. The nerves were cut near the origin of serratus ventralis muscle and a small cotton loop was tied on the central crushed end. The left splanchnic nerve was exposed through a cut in the left flank and freed in its length retroperitoneally from coeliac ganglion to diaphragm. After cutting across the ganglion a small cotton loop was tied on the central crushed end. The left sympathetic chain was exposed retropleurally in the chest at the level of the 7th or 8th rib, after removing the head and a part of the shaft of the rib. The chain was cut and freed caudally for about 1 cm, tying a cotton loop on the crushed upper end of the caudal portion of chain.

For stimulating or recording from nerves, stainless-steel wire electrodes were used. One wire was hooked and the nerve was held on it by the cotton loop wetted with 0·9% saline, while the other wire touched the nerve a suitable distance away. In this way the splanchnic and an intercostal nerve could be stimulated, and nerve volleys could be recorded off the thoracic sympathetic chain and off an intercostal nerve. Action potentials of trunk muscles were recorded through concentric needle electrodes or by two-wire stainless-steel electrodes with the wires touching the surface of the exposed muscle (Fig. 1). The tissues were kept warm and moist by pulling up the skin edges to contain a pool of warm paraffin oil.

Nerve and muscle action potentials were recorded through a.c.-coupled amplifiers feeding into a twin-beam oscilloscope unit. Nerves were stimulated with square voltage pulses synchronized with the oscilloscope time base, by means of an isolating transformer coupling.

RESULTS

Single shock stimulation of the central cut end of the splanchnic nerve may evoke a double discharge into lower intercostal and upper lumbar nerves. These two discharges are each prolonged and asynchronous, and are distinguished by their latencies, the second or late reflex having a total latency of the order of 60–80 msec (Fig. 2). Of the centrifugal impulses in the intercostal nerves certainly some go to trunk muscles. Early and late responses can be seen in the electromyogram (e.m.g.) of exposed muscles, such as the latissimus dorsi, external and internal obliques and transversus, the intercostals and the cutaneus maximus muscle.

The double response has been seen following single centripetal volleys in the splanchnic nerve of decerebrated and Dial-anaesthetized rabbits. It is not a feature of single centripetal volleys in an adjacent intercostal nerve, this producing only a single short-latency response.

Mutual independence of the responses. The late reflex was recorded as an efferent volley in an intercostal nerve when the decerebrated rabbit was paralysed by tubocurarine 1 mg/kg body weight I.V. It was also seen as a very localized response of muscle when anaesthesia was deep enough to block all but a trace of reflex activity in trunk muscles, and there was no apparent movement of the animal. In some preparations the late reflex

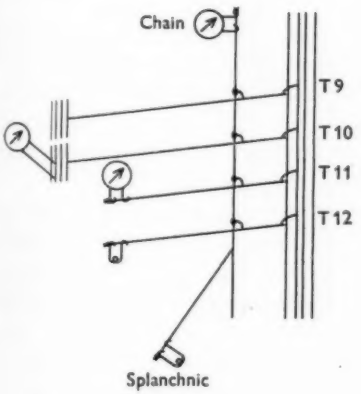

Fig. 1

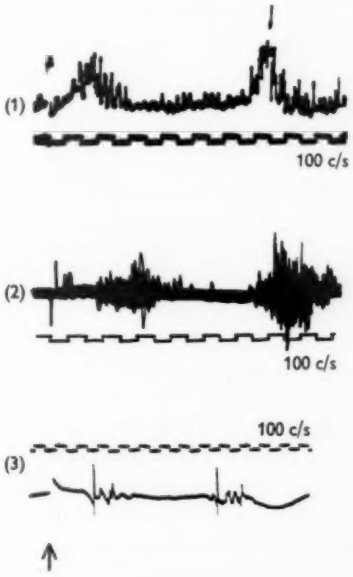

Fig. 2

Fig. 1. Diagram of experimental arrangements. Stimulating electrodes on central cut ends of splanchnic and 12th intercostal nerves. Recording electrodes on central cut end of 11th intercostal for recording reflex discharges, on rostral cut end of caudal portion of severed thoracic sympathetic chain for recording sample of centripetal splanchnic volley as chain electroneurogram, and on exposed trunk muscles for e.m.g.

Fig. 2. (1) Double discharge recorded off intercostal nerve after single-shock stimulation of splanchnic nerve. (2) Double response of m. ext. obliquus recorded as 12 superimposed records, electrodes touching exposed muscle about 1·5 cm apart. (3) Motor unit e.m.g. recorded by wire electrodes close together on surface of m. ext. obliquus, firing during early and late reflex. Stimulus artifact in line of arrow.

response was seen without the first, while in others a good early reflex alone was seen. In nerve-blocking experiments (see below) the early reflex was lost yet the late reflex remained. There was no predictable relationship between the sizes of the early and late reflexes. It is clear that the late response can occur independently of the early response and is not a consequence of some event, e.g. muscle movements, initiated by the early response. When the early reflex is absent, the late reflex may be mistaken for it unless the long latency is noted or the early reflex is brought up by post-tetanic potentiation (p. 471).

Spinal reflex nature of the responses. The discharge into the intercostal nerves and trunk muscles could conceivably be dependent upon some pathway lying outside the spinal cord, connecting splanchnic and sympathetic afferents to the somatic pathways. To check this, in an anaesthetized rabbit the left sympathetic chain was cut above T8 ganglion and dorsal roots T8 to T12 inclusive exposed by wide laminectomy. After confirming the presence of the double reflex, the dorsal roots were cut. The double response was completely absent although the upcoming volley in the sympathetic chain was unchanged.

The two responses are both mediated by arcs complete within the spinal cord. They have been recorded after spinal transections at C1 and at T1 levels. It was noted that in the decerebrated rabbit the spinal transection was usually followed by a decrease in the size of the reflexes, in contrast to the cat in which under similar circumstances there is an increase in size and lowering of threshold of the reflex (Downman, 1955).

Latency of e.m.g. unit activity. By carefully adjusting the recording electrodes on the muscle, single unit action potentials could be recorded, recognized by their constant form, size and duration. The same unit fired during both reflex responses. At the same site in the muscle or in other muscles units could be found which fired during only one or other of the reflexes. In one experiment a series of 165 consecutive records on maximal splanchnic stimulation were photographed, and a histogram constructed relating latencies to number of firings at that latency (Fig. 3). The resultant plot shows the wide fluctuations in latency of a unit firing during both responses.

Pattern of ascending splanchnic volley. The single shock to the splanchnic nerve sets up a volley which ascends the sympathetic chain and can be recorded off the cut end of the chain at T7 or T8 level. This volley consists of orthodromically-conducted impulses in afferent paths and antidromically-conducted impulses in motor paths. The volley seen in the rabbit chain is distinguished by the small A-fibre component, with a large B-fibre component. Well separated from the B volley is a large C-fibre volley (Fig. 4). This last wave may be markedly diphasic, even when the other

waves are monophasic, unless the cut end of the chain has been crushed many times or treated with a drop of 10% cocaine HCl solution. The volley as it is recorded off the chain in the rabbit differs from that seen in the cat, particularly in the larger size of the recorded C volley. The rabbit splanchnic nerve and sympathetic chain have very few large myelinated

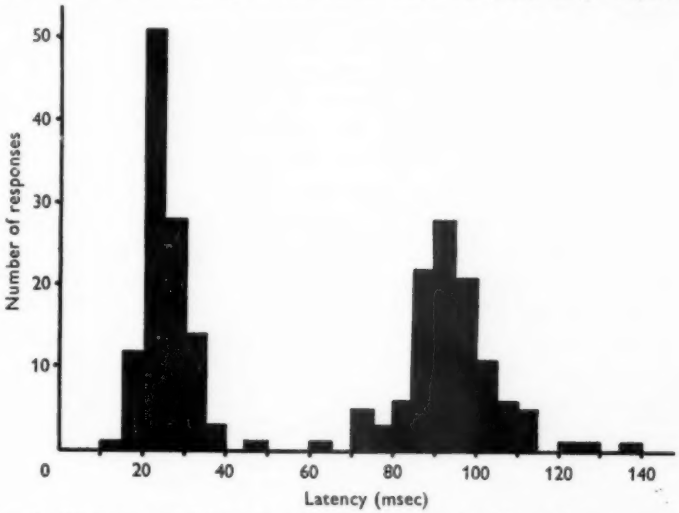

Fig. 3. Histogram of firing of motor unit of m. ext. obliquus in response to 165 consecutive stimulations of splanchnic nerve. Number of responses within each 5 msec period plotted against their latency in msec.

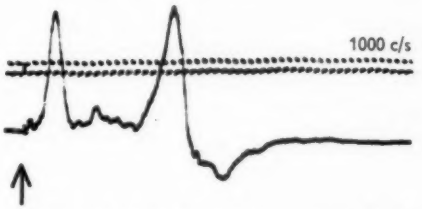

Fig. 4. Recording from cut end of thoracic sympathetic chain of volley set up by maximal shock to splanchnic nerve. Isolated preparation, suspended in warm oxygenated paraffin oil, 37·5° C; stimulus at arrow; conduction distance 28 mm.

fibres (Bishop & Heinbecker, 1930; Simpson & Young, 1945), and in the fine nerve strand the C-volley is well recorded. There is much evidence that splanchnic afferent fibres do enter dorsal roots; also that dorsal roots do contain C fibres (Ranson & Billingsley, 1916; Erlanger & Gasser, 1930; Gasser, 1950, 1955). It was noted that the splanchnic-to-chain volley recorded ascending had the same form as the chain-to-splanchnic volley

recorded descending, showing that there was no significant number of ganglionic relays in the pathway used. Also the ascending splanchnic-chain volley was of the same form and relative sizes at T7 and at T13 level, indicating that the sample of the volley recorded off the chain is comparable to that recorded lower down and containing the dorsal root entrant component. It is assumed that the recorded volley is a representative sample of the orthodromic afferent volley which enters the dorsal roots through the still connected white rami and spinal roots. Erlanger & Gasser (1930) have recorded C-fibre volleys in lumbosacral dorsal roots on stimulating leg nerves. However, in the present experiments, attempts to record the input volley in dorsal rootlets themselves have failed because the spontaneous background discharge completely swamps any trace of a synchronous volley in the few splanchnic afferents to be found in any one root. The splanchnic volley was recorded off a white ramus communicans connecting chain and spinal roots at T9 level. The volley components are the same though the C volley was smaller and more asynchronous. The evidence which is presented below can, however, best be explained on the assumption that the effective afferent volley is represented by the recorded sample.

Correlation between reflex discharges and splanchnic volley pattern

By recording the reflex discharges and the splanchnic volley at the same time, the two discharges can each be related to the different volleys in the electroneurogram. The following results have been recorded.

Threshold differences. The early reflex discharge is evoked by a stimulus which activates the myelinated fibres, A and B, yet is below the C threshold. Increasing stimulus voltage and/or duration elicited both a C volley in the chain and a late reflex discharge, these two responses reaching a maximum together. In some animals the threshold of this reflex was the same as the threshold of the C volley, with the duration of stimulus (0·5 msec) used. In other animals the threshold of C fibres was lower, and some C-fibre activity appeared below the stimulus maximum of the B volley. In these cases the early reflex and AB volleys had reached maximum before the advent of the late reflex (Fig. 5).

Differential block by 'gating'. By using the 'gating' method described by Kuffler & Vaughan Williams (1953) the myelinated volleys could be blocked. For this the stimulus anode was placed proximally on the splanchnic nerve, and interelectrode distance (approx. 8 mm), stimulus voltage and duration were adjusted until the faster volleys were blocked at the anode but the slower C volley passed, the volley being monitored as a chain electroneurogram. Effective block of the myelinated fibres was accompanied by loss of the early reflex, but the late reflex remained.

Differential block by induction coil shocks. The myelinated fibres could be differentially blocked by the method of Bishop & Heinbecker (1935*a*). A brief burst of shocks (< 1 sec duration) from an induction coil at approximately 40/sec was applied through a second pair of electrodes touching the exposed splanchnic nerve proximal to the stimulating pair.

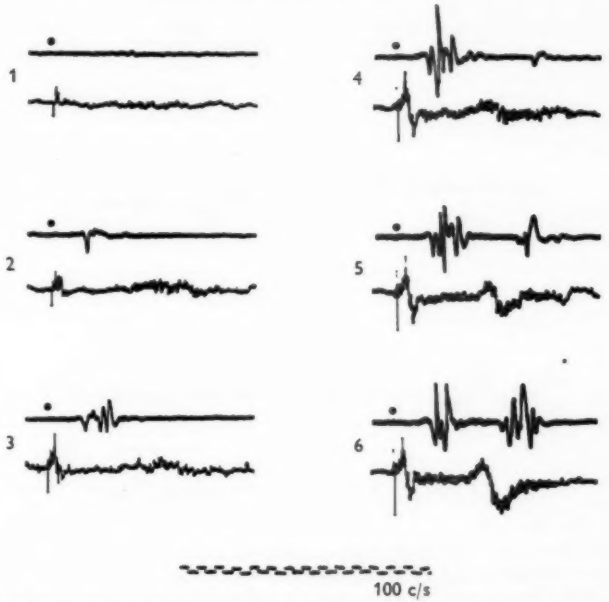

Fig. 5. Effect of increasing strength of stimulus applied to the central cut end of splanchnic nerve. Reflexes recorded as muscle activity; stimulus = square voltage pulse, 0·5 msec duration. Each pair of traces shows: top line, e.m.g. of m. ext. obliquus, bottom line, thoracic sympathetic chain electroneurogram. Trace 1 shows only small fast-fibre volley in chain. Traces 2, 3, show increasing size of AB wave with increase of early reflex. Traces 4, 5 show onset of late reflex at higher threshold, and its increase in size to maximum with maximum C volley in trace 6. It is notable that some C activity is evident even at low threshold of stimulus, trace 2 onwards.

A suitable strength and duration of the burst was found by gradually approximating the primary and secondary coils, until the stimulating action of the burst was followed by a block of conduction of the myelinated fibres for many minutes, but the C volley remained unchanged (Fig. 6). The early reflex was lost but the late reflex remained.

Lignocaine block. Placing a small drop about 1 mm diameter, of lignocaine HCl 0·1 % in 0·9 % NaCl solution upon the splanchnic nerve proximal to the stimulating electrodes sometimes blocked the C volley

without appreciably reducing the AB volley. This was accompanied by loss of the late reflex, with retention of the early reflex.

Conduction velocity of reflexogenic afferents. In these experiments the stimulating electrodes were moved a measured distance along the splanchnic nerve, thereby varying afferent conduction time. A reflex response set up by slower conducting afferents will show a greater change in latency than one set up by faster afferents. It was found that the latency of the late reflex response changed more than that of the early reflex.

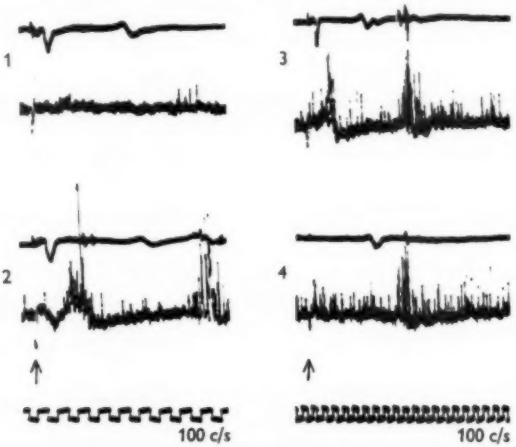

Fig. 6. Effect of short bursts (< 1 sec) of induction coil shocks applied on splanchnic nerve proximal to stimulating electrode. Each oscilloscope trace shows: top line, sympathetic chain electroneurogram showing splanchnic volley sample; bottom line, reflex discharges into 11th intercostal nerve. Arrow indicates maximal splanchnic stimulus. Time markers 100 cycles. Trace 1, initial responses. Trace 2, effect of burst of induction shocks which did not block myelinated fibres; potentiation of both early and late reflexes. Trace 3, as trace 2, to act as control for trace 4. Trace 4, taken after applying induction-coil shocks in a burst long enough and strong enough to block myelinated fibre volleys.

In a particular experiment (Fig. 7), moving the stimulating electrode 12·0 mm altered late reflex latency by 9·0 msec. This showed that the fastest reflexogenic afferents evoking the late reflex conducted at 1·3 m/sec. In another experiment the figure was 1·0 m/sec. This places the fibres within the conduction velocity range of the non-myelinated fibres.

During all experiments it was noted that the late reflex was never seen without a C volley in the chain record. However, a C volley might be recorded in the chain without an accompanying late reflex. Presumably this depended upon the conductivity of the spinal reflex arcs.

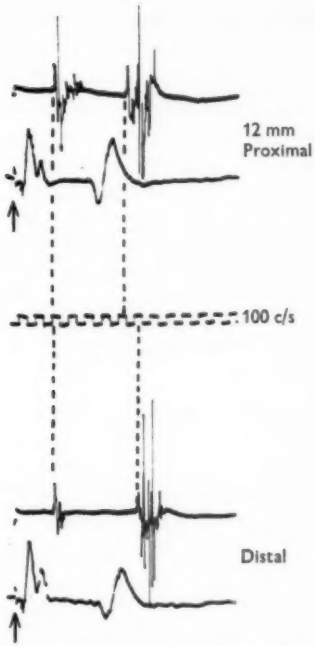

Fig. 7. Result of moving stimulating electrode 12 mm along splanchnic nerve. Each pair of oscilloscope traces shows above (retouched) e.m.g. of m. cutaneus maximus; below, splanchnic volley recorded in thoracic sympathetic chain. Arrows indicate splanchnic stimulus.

Facilitation of the reflex responses

Post-tetanic potentiation. Repeated stimulation of the splanchnic nerve was followed by increase of both early and later reflexes. It was, seen, for example, after short bursts of induction coil shocks (at 45/sec for < 1 sec) which stimulated, yet failed to produce block of the fibres (Fig. 6). This effect is not due to peripheral phenomena. Although Brown & Holmes (1956) have described post-tetanic enhancement of the C volley in sympathetic paths, it was not seen in the chain electroneurogram, although the majority of the fibres conducted at under 1·1 m/sec. When the C volley was altered it showed a small increase in latency with small decrease in height. In separate tests for post-tetanic enhancement, 1000 stimuli at 50/sec were given to splanchnic-chain preparations without increase of volley height. This was true both of the chain in the living animal, and of the chain isolated and suspended in warm oxygenated Ringer–Locke solution. In these tests the length of pathway used (60–80 mm) was

adequate for the demonstration of enhancement, according to Ritchie & Straub (1956). The method used was adequate, since enhancement was shown by rabbit hypogastric nerve under similar conditions.

Splanchnic-intercostal facilitation. In the cat it has been shown that afferent volleys in a splanchnic or an intercostal nerve may each evoke reflexes in another intercostal nerve. Two-volley tests have shown that a splanchnic afferent volley can facilitate or inhibit the reflex response set up by a subsequent intercostal afferent volley. The interaction seen depends on the central neurological status of the preparation (Downman, 1955; Downman & Hussain, 1958). Such interactions are also seen in

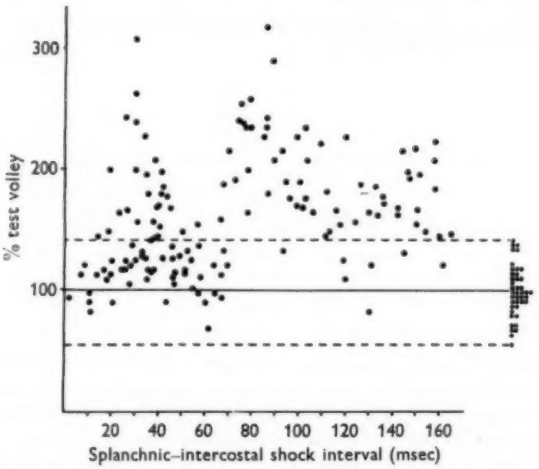

Fig. 8. Facilitation of intercostally-evoked test reflex response by prior conditioning splanchnic stimulation. Reflexes recorded off 10th intercostal nerve, stimulation of 11th intercostal nerve and splanchnic. Conditioned intercostal reflex discharge, measured as height of recorded volley, is expressed as percentage of unconditioned intercostally-evoked reflex. Range of control unconditioned responses shown by histogram on right, each spot representing a reflex, the interrupted horizontal lines indicating maximum variation of controls. Splanchnic-intercostal stimulus intervals in msec by which splanchnic precedes intercostal stimulus.

the rabbit. The maximum centripetal splanchnic volley provokes two phases of increased excitability in the spinal arcs. These were defined by a testing intercostally-evoked reflex which was elicited at various intervals after the splanchnic-evoked reflexes. Height of the test reflex was plotted, expressed as a percentage of the height of the unconditioned test reflex, against stimulus intervals. The plot shows two peaks, corresponding to the early and late reflex discharges set up by the splanchnic volley (Fig. 8).

The second phase of facilitation may last beyond the peak of the late reflex discharge. This may be related to the prolonged after-discharge of the motoneurones, which may last up to 500 msec for a maximal splanchnic stimulus (Fig. 9).

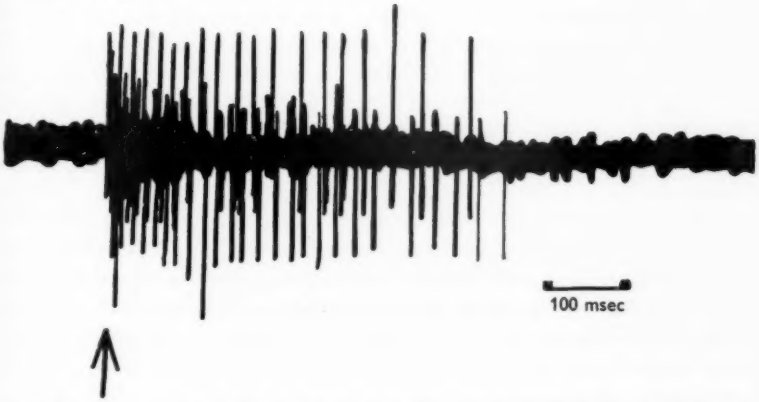

Fig. 9. Repetitive firing of motor units in response to single maximal shock to splanchnic afferent fibres, m. ext. obliquus (retouched).

DISCUSSION

The double reflex discharge which has been recorded in rabbits from thoracic motoneurones after single shock stimulation of the splanchnic nerve seems to be a feature of splanchnic-evoked reflexes only. Centripetal volleys reaching the same motor nuclei along intercostal nerves provoke only a short-latency discharge. The splanchnic-evoked double reflex may represent an unusual firing pattern of the motor nuclei, because it is most unlikely that they will in normal circumstances ever be bombarded by such synchronized splanchnic volleys. It was considered, however, that investigation of the response pattern could throw light upon the properties of central and peripheral splanchnic pathways.

It is notable that the volley set up in the rabbit splanchnic nerve by a single maximal shock shows a C fibre wave which is very large when one compares the relative sizes of A, B and C volleys. It is relatively much larger than in the cat splanchnic-chain preparation under similar recording circumstances.

Bernhard (1945) has reported that multiple efferent discharges in response to single shock stimulating can be related to internuncial activity rather than to afferent fibres of different conduction velocity. In this present work, however, evidence is presented showing that the second, long-latency reflex is set up by a slowly conducted synchronous

C-fibre volley. This correlation has been dependent upon sampling the centripetal splanchnic volley from the cut thoracic sympathetic chain into which it passes, the sampling being done off a portion of the chain above the level of entry of the effective afferent fibres. It is not, strictly, a record of the reflexogenic volley itself. The splanchnic shock will stimulate all fibres, setting up both orthodromic and antidromic volleys which will enter into the sample. The three lines of evidence—stimulus thresholds, differential block of myelinated fibres, and afferent conduction velocity of the effective volley—by which the late reflex has been related to the C-fibre volley, leaves little doubt that the relation will hold true in the roots themselves.

The late reflex discharge is mediated by spinal arcs. It is not dependent upon the early reflex which precedes it, nor is it secondary to some response in the animal to the early reflex. In some preparations the second discharge has been seen in nerve or muscle with no early discharge and this situation can be produced by the differential blocking of the myelinated fibres. One reflex which could stimulate afferent nerve endings to evoke another reflex is the jerk of the trunk muscles themselves. It was shown, however, that the late reflex into nerve survives the paralysis of the muscles by tubocurarine. Of the correlation between late reflex and C volley, it can be said that the late reflex has not been seen without a C volley in the splanchnic chain volley. On the other hand, a C volley in the chain may or may not evoke a late reflex, depending on the condition of the animal.

Besides evoking a double discharge, the splanchnic afferent volley evokes two phases of facilitation of the motoneurones. This was shown by using an afferent volley in another intercostal nerve as the testing stimulus. The peaks of these phases coincide with the times of maximum discharges. Each phase, like each discharge, can be related to the different fibre groups in the splanchnic nerve. The C-fibre volley has its facilitating action upon motoneurones which are fired in the short-latency intercostally-evoked test reflex. This shows that the two pathways overlap in some place, so that the C-fibre impulses can bring into subliminal fringe the same motoneurones which are fired in the early reflex. The recording of single motor units of muscle firing in both reflexes also shows that the C-fibres can activate the same motoneurones as myelinated fibres. It is remarkable that in some preparations, or even in different muscles or parts of a muscle of the same preparation, either of the reflex discharges may be present without the other. The central site and causes of the changes which may block the two pathways remain to be determined. There do occur considerable moment-to-moment fluctuations of excitability of the central reflex arcs. This is seen both in the fluctuations

of size and latency of reflexes and in the varying facilitation by a conditioning volley. These fluctuations are entirely central, the recorded splanchnic-chain volley remaining unchanged. This unpredictable fluctuation has been one of the puzzling features of these experiments; further work should show whether it is wholly of cord origin. Spinal transection above the arcs in the decerebrate animal did not cause the release of the reflexes described in the cat (Downman, 1955). Whatever the mechanism of suppression of the reflexes it can be overcome temporarily by facilitatory conditioning volleys in the same (i.e. splanchnic) or other (i.e. intercostal) afferent fibres. Repeated stimulation of the splanchnic nerve, when the reflexes are small or absent, causes increase in size. Induction coil shocks at 40/sec and below blocking strength caused prolonged post-tetanic potentiation. This was not accompanied by any sign of post-tetanic enhancement of the C volley in the peripheral pathway as described by Brown & Holmes (1956) for other nerves, although the majority of fibres in the volley conduct at under 1·1 m/sec, which was the upper limit for size of the fibres showing enhancement set by Brown and Holmes. Failure to record enhancement was not a failure of the method since it could be recorded on isolated rabbit hypogastric nerves.

Of three lines of evidence defining C volleys as reflexogenic, the least reliable was the use of thresholds. The maximum C volley required stimulus durations of 0·5 msec. At these durations the threshold of the lowest threshold C fibres approaches that of B fibres. Bishop & O'Leary (1939) found in rabbits that the most irritable C fibres respond at lower threshold than the least irritable B fibres. This finding has been confirmed. It should be noted that Bishop & Heinbecker (1935*b*), in a paper on the responses of mammalian nerve to strong shocks, describe random repetitive discharge of myelinated fibres. This lasted a large fraction of a second after a single shock over C-fibre threshold to the vagus. We have recorded such a discharge in isolated chain preparations. The second reflex response may be facilitated by this discharge, but is not set up by it. The induction-coil blocking method blocks conduction of both the initial volley and the repetitive discharges along the splanchnic nerve myelinated fibres. Gating is a less distinctive method since the block, being effective only during the stimulus pulse, does not block transmission of any repetitive discharge. In addition to the evidence of reflex thresholds, the other lines of evidence —differential blocking and conduction velocity—show that the C volley is effective. Within this volley the fastest reflexogenic fibres conduct more slowly than the fastest C fibres in the chain. Whether the larger C fibres are ineffective because of lack of central connexions or lack of numbers is not known.

SUMMARY

1. Single shock-stimulation of the central cut end of the splanchnic nerve in rabbits, after decerebration or under Dial anaesthesia, evokes a double reflex discharge.

2. This double reflex is seen as an early short-latency discharge followed by a later long-latency (60–70 msec) discharge into intercostal nerves and trunk muscles.

3. The late reflex is set up by the afferent volley in the C fibres of the splanchnic nerve. The correlation between the late reflex and the afferent C volley has been demonstrated by (*a*) stimulation threshold, (*b*) differential blocking of myelinated fibres, and (*c*) estimation of the conduction velocity of the reflexogenic volley.

4. The splanchnic volley provides two phases of facilitation of intercostal reflex activity, tested by an intercostally evoked reflex, corresponding in timing to the double reflex discharge

5. Post-tetanic potentiation of both reflex discharges occurs without post-tetanic enhancement of the splanchnic chain volley.

6. Motor units may fire during both reflex discharges.

7. The great fluctuation of conductivity in the arcs examined during these experiments is emphasized.

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